IMMUNOSTIMULATORY ORAL MICROBIOME IN HEALTH, INFLAMMATION, AND AUTOIMMUNE DISEASES

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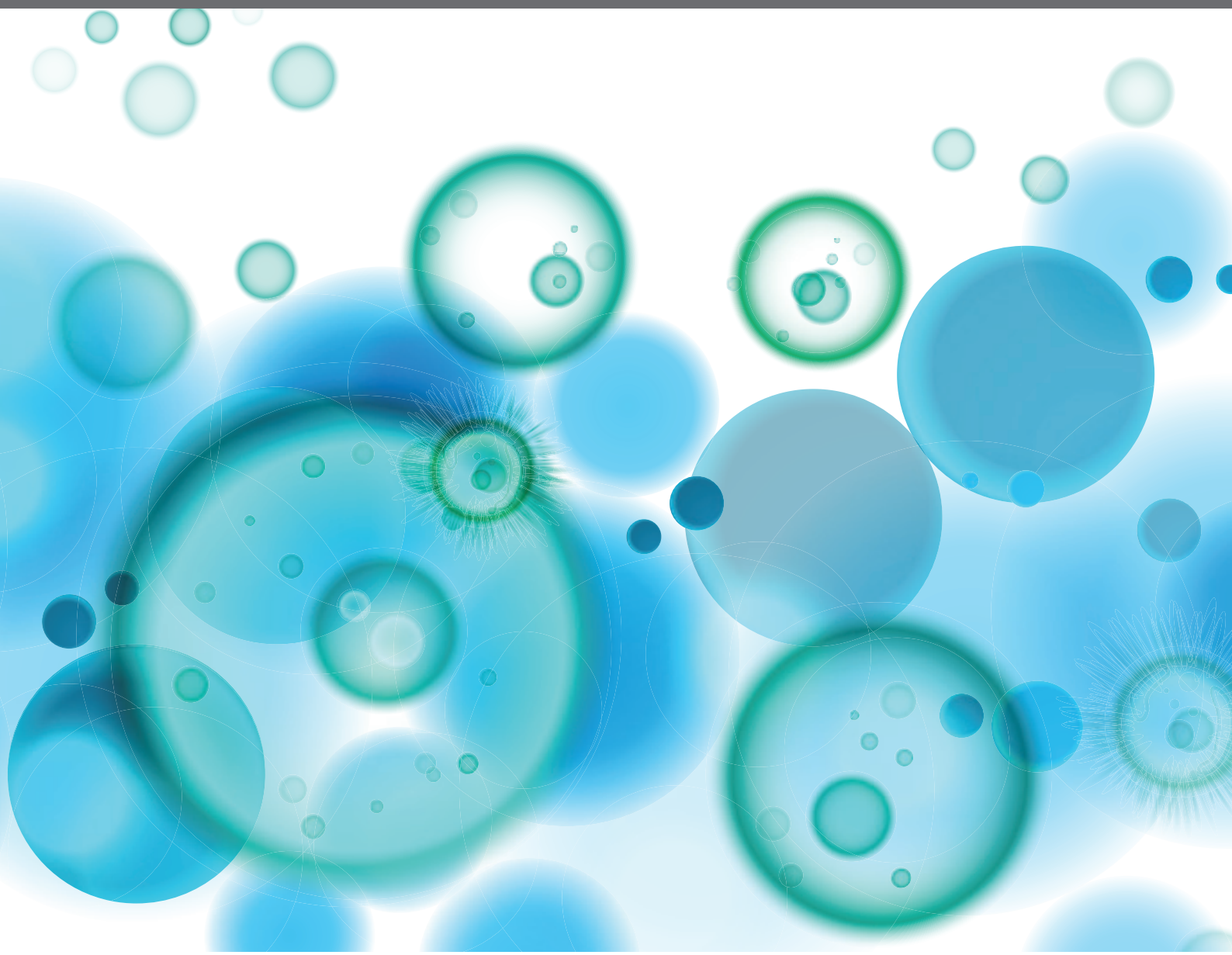

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IMMUNOSTIMULATORY ORAL MICROBIOME IN HEALTH, INFLAMMATION, AND AUTOIMMUNE DISEASES

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REVIEW
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The Butyrogenic and Lactic Bacteria of the Gut Microbiota Determine the Outcome of Allogenic Hematopoietic Cell Transplant

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Graft versus host disease (GVHD) is a post-transplant pathology in which donor-derived T cells present in the Peyer's patches target the cell-surface alloantigens of the recipient, causing host tissue damages. Therefore, the GVHD has long been considered only a purely immunological process whose prevention requires an immunosuppressive treatment. However, since the early 2010s, the impact of gut microbiota on GVHD has received increased attention. Both a surprising fall in gut microbiota diversity and a shift toward Enterobacteriaceae were described in this disease. Recently, unexpected results were reported that further link GVHD with changes in bacterial composition in the gut and disruption of intestinal epithelial tight junctions leading to abnormal intestinal barrier permeability. Patients receiving allogenic hematopoietic stem cell transplant (allo-HCT) as treatment of hematologic malignancies showed a decrease of the overall diversity of the gut microbiota that affects *Clostridia* and *Blautia* spp. and a predominance of lactic acid bacteria (LAB) of the *Enterococcus* genus, in particular the lactose auxotroph *Enterococcus faecium*. The reduced microbiota diversity (likely including Actinobacteria, such as *Bifidobacterium adolescentis* that cross feed butyrogenic bacteria) deprives the butyrogenic bacteria (such as *Roseburia intestinalis* or *Eubacterium*) of their capacity to metabolize acetate to butyrate. Indeed, administration of butyrate protects against the GVHD. Here, we review the data highlighting the possible link between GVHD and lactase defect, accumulation of lactose in the gut lumen, reduction of Reg3 antimicrobial peptides, narrower enzyme equipment of bacteria that predominate post-transplant, proliferation of *En. faecium* that use lactose as metabolic fuels, induction of innate and adaptive immune response against these bacteria which maintains an inflammatory process, elevated expression of myosin light chain kinase 210 (MLCK210) and subsequent disruption of intestinal barrier, and translocation of microbial products (lactate) or transmigration of LAB within the liver. The analysis of data from the literature confirms that the gut microbiota plays a major role in the GVHD. Moreover, the most recent publications uncover that the LAB, butyrogenic bacteria and bacterial cross feeding were the missing pieces in the puzzle. This opens new bacteria-based strategies in the treatment of GVHD.

Keywords: microbiota, butyrate, lactase, lactose, cross-feeding, graft versus host disease, hematopoietic cell transplant

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THE GUT MICROBIOTA

About 100 trillion bacteria present in the intestinal lumen (especially the colon) compose the human gut microbiota. During the last two decades, the advanced methods of high throughput sequencing and culturomics (Lagier et al., 2016) have highlighted the enormous diversity of bacteria found in humans. With its considerable bacterial genetic diversity, over 1,000 species and 7,000 strains identified, the human gut microbiota is a quite complex ecosystem, in which the phyla Firmicutes (species such as *Lactobacillus*, *Enterococcus*, and *Clostridium*) and Bacteroidetes (species such as *Bacteroides*) account for the majority of species. Other phyla including Proteobacteria (*Escherichia coli*), Actinobacteria (Bifidobacteria), Cyanobacteria, Fusobacteria, and Verrucomicrobia are also present in lower abundance (**Figure 1**; Eckburg et al., 2005; Qin et al., 2010). Microorganism DNA represents about 90% of the total DNA found within human bodies (Human Microbiome Project Consortium, 2012).

The capacity to rapidly identify a large number of bacterial species in human microbiota opened the way to compare the gut bacterial composition in cohorts of individuals with metabolic or infectious diseases to healthy controls in search of beneficial and non-beneficial bacteria (Million et al., 2016; Tidjani Alou et al., 2017; Elkrief et al., 2019).

During the embryo development (between the first and third trimester of pregnancy), there are shifts in maternal microbiota composition, which likely provide advantages to the fetus survival (Koren et al., 2012). It is usually admitted that the microbial colonization occurs first in the amniotic fluid and placenta, and then in the maternal gut microbiota which supports the development of a prenatal microbiota in the fetus (Collado et al., 2016). When the *in utero* development is achieved, the fetus migration through the vagina favor a bacterial transfer between the microbiota colonizing the birth canal of the mother and the fetus. In favor of an early bacterial colonization of the fetus, it was observed that *Es. coli*, *Enterococcus faecalis*, and *Staphylococcus epidermidis* were isolated from the

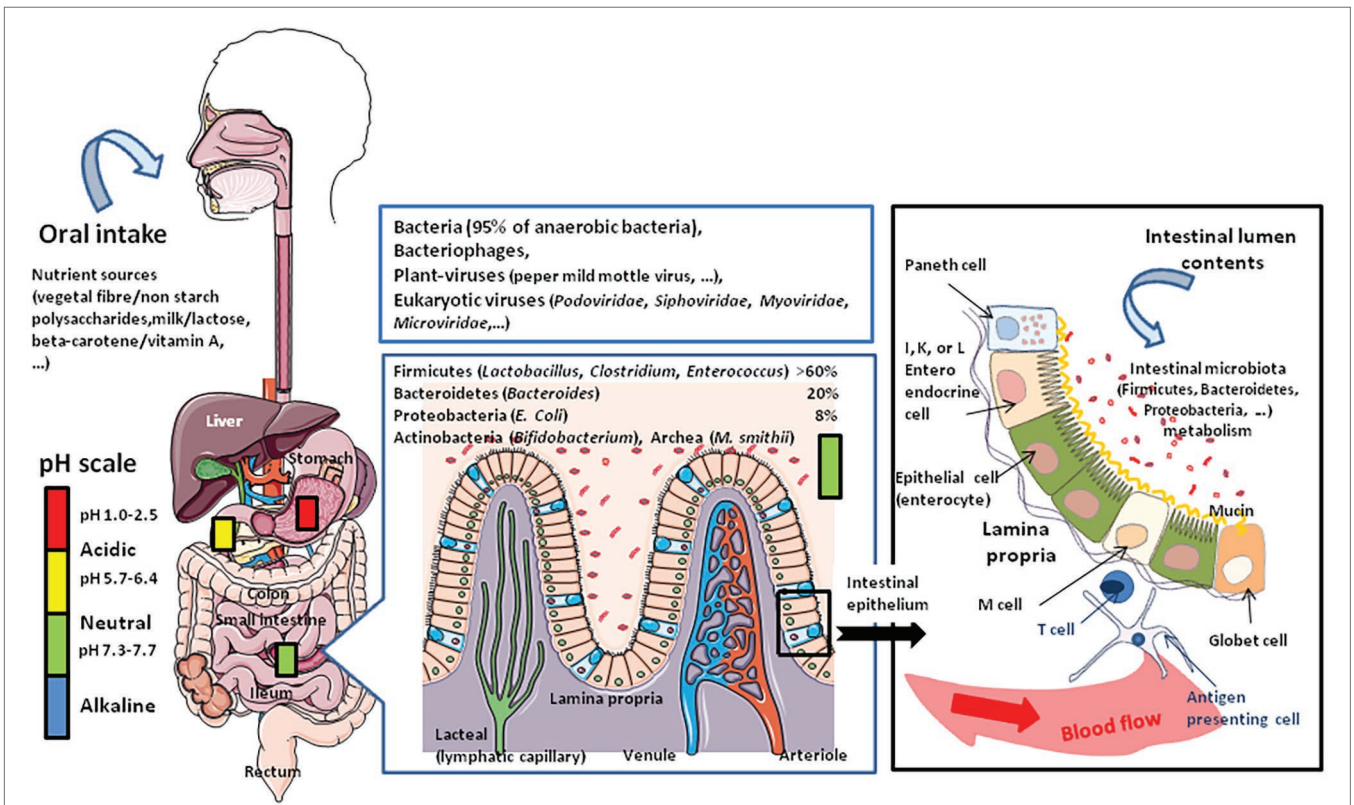

FIGURE 1 | Schematic representation of oral intake including milk that contains water, vitamins, minerals, and biologically active molecules such as adipokines, leptin, adiponectin, and growth factors such as epidermal growth factor and fibroblast growth factor 21 (FGF21). The nutriments first meet the stomach microenvironment. The figure illustrates the variations in pH with marked changes from acidic in the stomach to almost neutral or slightly alkaline in the intestine. The diversity of microbiota increases from the stomach to duodenum, small intestine, and colon, where the concentration of bacteria is the highest. The abundance of microbes is about 10^1–10^3 colony forming unit (CFU) per gram in the stomach and duodenum, 10^4–10^7 CFU/g in the small intestine, and 10^{10}–10^{11} CFU/g in the colon. The **middle panel** illustrates the microbiome and virome diversity in the intestinal microenvironment. The **right panel** illustrates the physiological organization of the intestinal epithelium during homeostasis. The epithelium (composed of epithelial cells) is protected by a mucus layer synthesized by goblet cells which secrete the mucins (e.g., MUC2 – mucin gel). The mucus layer allows nutrients to transport to epithelial cells but prevents bacteria attachment. The commensal intestinal microbiota is limited to the epithelium-distal mucus layer, while the epithelium-proximal mucus is largely devoid of bacteria. Intestinal bacteria such as *Bacteroides* and *Clostridia* ferment lactose into gases, hydrogen carbon dioxide, and methane when the microbiota contains an abundance of Archaea.

meconium of healthy neonates (Nicholson et al., 2012). The neonate survival and growth next depends on mother milk feeding that is his/her essential source of nutrients and that contributes to shape his/her gut microbiota. During the early period of breastfeeding, the infant's gut is characterized by a low microbiota diversity and become colonized by beneficial bacteria such as *Bifidobacterium*, *Lactobacillus*, and *Prevotella* (Rautava et al., 2012; Arrieta et al., 2014). The mother milk contains about 70 g/L (7%) of lactose produced by the mammary gland (lactose synthesis requires the enzyme galactosyl transferase, which combines activated uridine di-phosphate galactose with glucose), as well as numerous biologically active factors including growth factors. The lactose is well digested by newborns whose small intestinal brush border enterocytes produce lactase in abundance. Dietary intake and bile acids (steroid acids produced in the liver and whose main function is to facilitate the absorption of fat-soluble vitamins and cholesterol) determine at least in part the microbiota assembled during the first few years of life and a shift is observed in favor of anaerobic bacteria that contribute to produce many metabolites of fermentation such as production of short-chain fatty acids (SCFAs; e.g., acetate, propionate, and butyrate; Shanahan et al., 2017). By a mechanism of cross feeding, the intestinal symbiotic microbiota contributes to maintain the production of butyrate by butyric acid bacteria; they also participate to the inhibition of pathogens growth by competing consumption of nutriments and allow to prevent toxin translocation by decomposing metabolic carbohydrates to obtain SCFAs. These SCFAs act on enteroendocrine cells of the gut through heterotrimeric guanine nucleoside-binding protein (G-protein)-coupled receptors that secrete a variety of bioactive compounds.

Lactase production decrease in the majority of the world population after weaning and most healthy adults (67–75%) produce less, sometimes very little lactase (about 10% of the concentration found of neonatal levels), whereas 25–33% retain the ability to digest lactose into adulthood (Troelsen et al., 1997). The study of ileostomy effluent samples from adult patients provided evidence that the small intestine metagenome is enriched in genes related to carbohydrate metabolism compared to the fecal metagenome (Zoetendal et al., 2012), suggesting that carbohydrate metabolism is a central function of the small intestine with lactase and propionate fermentation activities encoded by many taxa from the ileal effluent, in particular *Streptococcus* that help the growth of secondary fermenters (e.g., *Veillonella* and *Clostridium*). It is also admitted that the small intestine microbiota is phylogenetically less diverse but more dynamic than that of the colon (Booijink et al., 2010). Many bacteria living in the intestinal tract such as *Lactobacillus* sp., *Bifidobacterium* sp., *Bacillus* sp., and *Es. coli* produce lactase and play a major function in lactose absorption in the colon (Rhimi et al., 2009; Juajun et al., 2011). Dietary fibers (non starch polysaccharides) escape digestion in the human small intestine and are then broken down into simple sugars by anaerobic bacteria in the caecum and colon. The colon is more equipped to degrade complex carbohydrates and its main function is absorption of water and electrolytes and storage of fecal matter before expulsion (Turnbaugh et al., 2010). Diet degradation and absorption is under control of metabolic processes that largely depend on expression of bacterial enzymes, which are a direct reflection of the gut microbiota. The intestinal microbiota produces essential vitamins such as vitamin K, B1, B6, B9 (folic acid), and B12. Bacteria from the Firmicutes, Bacteroidetes, and Actinobacteria phyla play a role in bile acids metabolism through bile salt hydrolase activity, which catalyzes the deconjugation of conjugated bile acids (cholesterol derivatives synthesized in the liver) to liberate free primary bile acids, upregulates mucosal defenses, and controls the cholesterol homeostasis (Jones et al., 2008).

It is currently well established that homeostasis and dysbiosis are largely influenced by the composition of gut microbiota and the balance existing between different strains of bacteria (Hooper and Macpherson, 2010; Littman and Pamer, 2011). Environmental selection pressure (e.g., over- or under-nutrition, and antibiotics; World bank, 2017; Vieco-Saiz et al., 2019) and competitive exclusion between bacteria (e.g., probiotics and pathogenic bacteria) are expected to be the major driving forces that shape the bacterial composition of the human gut microbiota (Walter and Ley, 2011; Devaux and Raoult, 2018). Here, we provide evidence that the microbiome (the microbiota and the bacteria enzymatic equipment-driven metabolome) determine the outcome of graft versus host disease (GVHD).

IMPACT OF THE GUT MICROBIOTA ON GVHD

If, for certain human diseases, the pathological process linked to change in the composition of the gut microbiota begins to be understood, for others it remains to be elucidated. Hematopoietic stem cell (HSC) transplantation remains indispensable for the treatment of several malignant disorders (Korbling and Freireich, 2011). Before the graft infusion, most protocols require killing of malignant cells (by chemotherapeutic drugs and/or radiotherapy myelosuppressive treatment) that cause a cytotoxic burst of tumor and normal immune cells associated with a pro-inflammatory status (Blazar et al., 2012). Then, cellular reconstitution is achieved by transplantation of peripheral blood stem cells that is the preferred source of allogenic HSC in adults in order to replace the hematopoiesis of the recipient by that of the donor. Next, it is of major importance to avoid graft rejection (mediated by the recipient immune cells) using immunosuppressive drugs. The differentiated immune cells from donor origin colonize the body (including the gut) with a risk of GVHD. The immunosuppressive therapies practiced in these patients incompletely control GVHD and increase susceptibility to infections. GVHD is considered to be mediated by activated $CD8^+$ cytotoxic T-cell (CTL) from the graft donor after these cells had met graft recipient alloantigens in the context of antigen presenting cells (APCs) in the subepithelial dome of gut Peyer's patches, the major sites where immune response is set up against luminal antigens and microorganisms (Murai et al., 2003). About 40–50% patients experience severe gastrointestinal damages from acute GVHD

that turn to be fatal in about 15% of allo-transplant recipients refractory to standard steroid therapy (Blazar et al., 2012; Köhler and Zeiser, 2019). The follow-up of chimerism in patients after allo-HCT allows quantification of the donor or recipient origin of cells obtained from blood or bone marrow samples, and the chimerism is considered complete when 95% of cells are phenotypically of donor origin.

Beside the immunological activation that has long been studied in allo-transplantation, the impact of gut microbiota on GVHD has received increasing attention over the recent years. Indeed, bacterial lipopolysaccharide (LPS) released from injured gut during the condition regiment taken by the patient was considered as responsible for initiating an innate immune response through activation of toll-like receptors (TLRs) and production of cytokines, serving as a breeding ground for the onset of GVHD (Hill et al., 2000). In humans, the treatment of hematologic malignancies by allo-HCT was characterized by a marked decrease of the overall diversity of the gut microbiota, *Enterococcus* domination, and the patients with the lowest gut microbiota diversity were those with the higher mortality outcomes (Taur et al., 2012, 2014; Andermann et al., 2018). Thus, the pathophysiological mechanisms implemented in the context of a post-transplant GVHD accompanied by a modification of the gut microbiota deserve further investigation. Early administration of large spectrum antibiotics depleting *Blautia* spp. (and at a lesser extend *Clostridia*) was associated with increased GVHD and higher mortality, whereas increased abundance in *Blautia* spp. improves survival (Jenq et al., 2015). It was next confirmed that the abundance of *Clostridia* decreased in the microbiota of allo-transplant patients that experienced GHVD and was accompanied by alteration in gastrointestinal microbiota-derived butyrate (Mathewson et al., 2016). Metabolites, such as 3-indoxyl sulfate that originates from the degradation of dietary protein-derived tryptophan to indole by the tryptophanase of intestinal commensal bacteria and that is known to enhance epithelial barrier integrity and to reduce inflammation (Bansal et al., 2010), may serve as a urine marker for monitoring GVHD since microbiota perturbation in patients with GVHD is associated with lower urine levels of 3-indoxyl sulfate (Weber et al., 2015, 2017). Although bacterial populations such as *Streptococcus*, *Gemella*, and *Veillonella* considered as genera defining the core oral microbiota were little affected by the allo-HCT, at the bacterial species level, it was reported that the oral microbiota was affected in patients who developed respiratory complication after allo-HCT (e.g., decrease in *Streptococcus infantis* and increase in *Veillonella parvula*; Ames et al., 2019). Since microbiota is connected and dysbiosis is likely to be generalized. The oral microbiota could therefore be considered as a new biomarker to monitor the allo-HCT evolution.

Recently, three independent research groups decipher the molecular mechanism linking GVHD to changes in the species composition of the gut microbiota (Golob et al., 2019; Nalle et al., 2019; Stein-Thoeringer et al., 2019). The analysis of the gut microbiota of a cohort of patients with allo-HCT revealed an increase in *Enterococcus faecium*, a lactose auxotroph bacteria (which requires lactose for its *in vitro* growth), in the patient's gut microbiota along with inflammation and intestine damages (Stein-Thoeringer et al., 2019). A decrease in *Clostridium* spp. accompanied by a significant reduction of fecal butyrate was also reported in the allo-HCT patients. This corroborate the results from Golob et al. (2019) who reported that a decrease in butyrogenic bacteria (e.g., *Blautia* spp.) in allo-HCT patients favor the GVHD, whereas administration of butyrate provide protection against GVHD.

Stein-Thoeringer et al. (2019) found that the sugar metabolism of the allo-HCT patients was impaired with an over-expression of enzymes involved in the degradation of lactose and galactose and that patients bearing a genetic polymorphism which decreases lactase expression (an enzyme also named β-D-galactosidase that is synthesized by enterocytes of the small intestine that break down the disaccharide D-lactose into D-galactose and D-glucose monomers) suffer from lactose absorption failure. It was hypothesized that lactic acid bacteria (LAB) *En. faecium* may possibly mediate the pro-inflammatory process (Zitvogel and Kroemer, 2019). Although patients with GVHD have increased intestinal permeability, the distribution of zonula occludens-1 (ZO-1) and actin were found unaltered and, in most cases, epithelial damages are limited to apoptosis of crypt epithelial cells associated with an over-expression of myosin light chain kinase 210 (MLCK210) and increased myosin II regulatory light chain phosphorylation (Nalle et al., 2019). Non-muscle myosin II (NMII), notably NMIIA, a key Rho kinase target, plays a role in epithelial cell-cell adhesion by controlling the local E-cadherin accumulation at the cell-cell contact (Priya et al., 2015). Moreover, E-cadherin is known for being used as target receptor for several bacteria and cleavage of E-cadherin by sheddases followed by the release of soluble E-cadherin is a mechanism frequently involved in disruption of the intestinal epithelium and invasive bacteria transmigration (Devaux et al., 2019). A higher risk of blood stream infection caused either by vancomycin-resistant *Enterococcus* or Gram-negative bacteria (e.g., *Es. coli* or *Klebsiella pneumoniae*) was reported, and post-engraftment vancomycin-resistant *Enterococcus* colonization was associated with increased mortality (Ford et al., 2017; Tamburini et al., 2018; Zama et al., 2020).

THE BUTYRATE PATHWAY

Recently, Golob et al. (2019) reported that the butyrogenic bacteria *Blautia* spp. were less abundant in allo-HCT who experienced GVHD, whereas administration of butyrate provided protection against GVHD. A decrease of fecal butyrate was also reported by Stein-Thoeringer et al. (2019) in the allo-HCT patients who experienced GVHD (**Figure 2**).

Butyrate, considered a protective molecule against inflammation is the end-product of anaerobic bacteria fermentation of non-digestible carbohydrates and also a component of dairy products (e.g., butter, milk, and cheese). The butyric acid bacteria are ubiquitously present in the gut microbiota of healthy humans. Oxidative stress sensitive bacteria include the butyrate producers, which mainly belong to the Firmicutes,

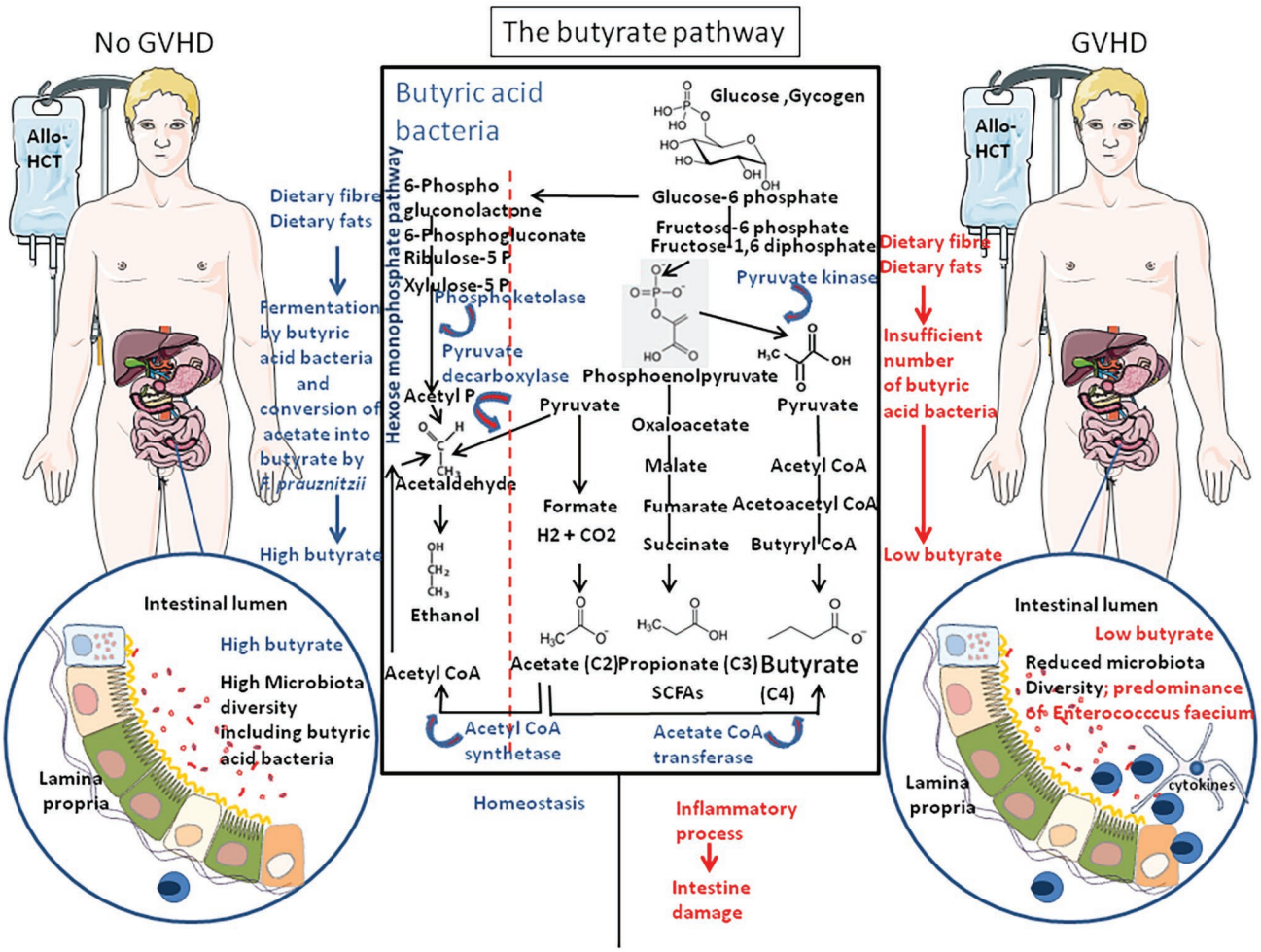

FIGURE 2 | The butyrate pathway. Most of the dominant species of human colon microbiota produce acetate during the fermentation of carbohydrates. Acetate, propionate, and butyrate are the main short-chain fatty acids (SCFAs) found in humans. The main pathway for acetate formation is the oxidative decarboxylation of pyruvate leading to the synthesis of an ATP molecule. In the human colon, propionate is mainly produced by the succinate decarboxylation pathway. Propionate is mainly synthesized by the species of the genus *Bacteroides*, *Propionibacterium*, and *Veillonella*. However, the synthesis of propionate *via* lactate (not shown), could also be important, especially in the case of diets rich in non-digestible oligosides. *Clostridium*, *Eubacterium*, *Faecalibacterium*, *Roseburia*, and *Butyrivibrio* have been shown to be capable, *in vitro*, of producing butyrate by the condensation of two molecules of acetyl-CoA with synthesis of one molecule of ATP. Acetyl-CoA is also the precursor of the synthesis of ethanol, produced *in vitro* by many species such as those of the genera *Bacteroides*, *Lactobacillus*, or *Bifidobacterium*. *Faecalibacterium prauznitzii* was found able to use acetate as a source of butyrate. Butyrate is a major metabolite in colonic lumen arising from bacterial fermentation of dietary fiber and has been shown to be a critical mediator of the colonic inflammatory response. Butyrate is a source of energy and it plays a critical role in homeostasis through its ability to counteract inflammation-mediated ulcerative colitis. Patients receiving allo-HCT as treatment for hematologic malignancies and who have an abundance of butyrate-producing bacteria **(left)** have the highest probability to get reed of carbon-dioxide (GVHD), whereas those with low abundance of butyric acid bacteria **(right)** are more often faced to GVHD.

Lachnospiraceae, Ruminococcaceae (e.g., *F. prausnitzii*, *Roseburia intestinalis*, *Eubacterium* spp., and *Coprococcus* spp.), and mucosa associated symbionts such as *Lactobacillus* spp. or *B. adolescentis*. These bacteria contribute to absorb dietary fats and lipid-soluble vitamins and facilitate lipid absorption and can digest polysaccharide dietary fiber and oxidize sugar to pyruvate. Different functionally distinct groups of butyrate-producing bacteria are present in the human large intestine: strains of *R. intestinalis* and *F. prausnitzii* possess butyryl CoA: acetate CoA transferase and acetate kinase activities but lack butyrate kinase, whereas the strain L2–50 of *Coprococcus* sp., possesses butyryl CoA: acetate CoA transferase, acetate kinase, and butyrate kinase (Duncan et al., 2002). This observation corroborates old studies performed on anaerobic butyric acid bacteria of cattle rumen that already demonstrated that among 48 strains, about 50% were able to hydrolyze starch and the number of strains that fermented certain carbohydrates was glucose, 48; esculin, 46; xylose, maltose, and cellobiose, 44; sucrose and salicin, 43; fructose, 42; lactose and inulin, 40; dextrin, 37; xylan, 34; trehalose, 16; and mannitol, 1, indicating major strain differences in genes coding for their enzyme equipment (Bryant and Small, 1956).

During the fermentation process, they produce SCFAs: acetate (C2), propionate (C3), and butyrate (C4). The SCFAs concentration is 10 times higher in the colon than in the ileum (Cummings et al., 1987). Acetate, propionate, and butyrate represent between 85 and 95% of total SCFAs found in the host and the molar ratio acetate/propionate/butyrate is about 60/20/20 (Cummings and Macfarlane, 1991; Topping and Clifton, 2001). About 95% of the SCFAs produced in the colon are absorbed from the colonic epithelium and 5–10% of the SCFAs are excreted by the feces (Hijova and Chmelarova, 2007). Acetate and propionate are transported through the blood to a variety of organs, where they are substrates for oxidation, lipid synthesis, and energy metabolism. Acetate is mainly metabolized by liver cells (50–70%), but also by the heart and skeletal muscles and the brain, making it an important source of energy. Propionate is also metabolized by the liver and used by hepatocytes for glucogenesis (Cummings et al., 1987; Nicholson et al., 2012). Butyrate is used by the cells of the colon and liver and targeted to their mitochondrial compartment for energy purposes (Clausen and Mortensen, 1994; Basson et al., 2000; Della Ragione et al., 2001).

Most of the dominant species of human colon microbiota produce acetate during the fermentation of carbohydrates. The main pathway for acetate formation is the oxidative decarboxylation of pyruvate leading to the synthesis of an ATP molecule. In the human colon, propionate is mainly produced by the succinate decarboxylation pathway (Miller and Wolin, 1979). Propionate is mainly synthesized by the species of the genus *Bacteroides*, *Propionibacterium*, and *Veillonella*. However, the synthesis of propionate *via* lactate could also be important, especially in the case of diets rich in non-digestible oligosides. Butyrate is mainly produced by distinct families within the Firmicutes, Ruminococcaceae, and Lachnospiraceae. But members of other phyla including Bacteroidetes, Actinobacteria, Fusobacteria, and Proteobacteria are potential butyrate producers (Vital et al., 2014; Zhang et al., 2019). Bacteria that belong to *Clostridium*, *Eubacterium*, *Faecalibacterium*, *Roseburia*, and *Butyrivibrio* have been shown to be capable, *in vitro*, of producing butyrate by the condensation of two molecules of acetyl-CoA with synthesis of one molecule of ATP. Most of the bacterial diversity in bacteria that produce butyrate by the acetyl-CoA pathway is associated with Ruminococcaceae and Lachnospiraceae, whereas other bacteria such as Bacteroidetes mainly use other (Glutarate, Lysine) pathways (Vital et al., 2014). Acetyl-CoA is also the precursor of the synthesis of ethanol, produced *in vitro* by many species such as those of the genera *Bacteroides*, *Lactobacillus*, or *Bifidobacterium*. Pyruvate is a central compound of the butyrate pathway and in turn can be oxidized to acetyl CoA with production of hydrogen, methane, and carbon dioxide gases (CO_2 increases the pH) and energy, which the bacteria require for growth and production of many molecules. Part of acetyl CoA is converted into acetic acid with ATP production and butyryl CoA that is metabolized into butyrate with ATP production. In acetate-rich diet, where acetate was delivered by resistant starch directly into the gastrointestinal tract, butyric acid bacteria can convert both exogenous and gut microbiota-produced acetate into butyrate (a reaction that requires acetate CoA transferase and butyrate kinase). One of the rare known bacteria that can achieve this enzymatic reaction is *F. prausnitzii* (Duncan et al., 2002), thereby contributing to the protection of the intestinal mucosa barrier and to the promotion of *Bifidobacterium* and *Lactobacillus* improving intestinal function (Si et al., 2018). *F. prausnitzii* grows poorly in culturomic medium that does not contain acetate and this requirement likely explains its dependence on rumen fluid. SCFAs are able to bind and activate the G-protein coupled cell surface receptors (including FFA2/GPR43 and FFA3/GPR41) expressed by enteroendocrine I, K, and/or L epithelial cells that are known to play an important role in the regulation of glucose homeostasis and appetite (Jorsal et al., 2018; Priyadarshini et al., 2019). In a murine model, it was shown that FFA2/GPR43 is also strongly expressed in a large population of leukocytes in the lamina propria; that FFA3/GPR41 is expressed in subpopulations of ghrelin and gastrin cells in the stomach, in secretin cells of the proximal small intestine, in GLP-1, YY, and neurotensin cells of the distal small intestine and proximal colon; and that a gradient of FFA3/GPR41 expression exists among the somatostatin cells from less than 5% in the stomach to more than 95% in the rectum (Nohr et al., 2013). FFA2/GPR43 has similar affinity for acetate, propionate, and butyrate whereas FFA3/GPR41 has preferential affinity for propionate, although it also binds butyrate. In a murine model of GVHD, mice orally force-fed with GPR43 antagonist GLPG0974 (10 mg/kg/day) demonstrated significant higher GVHD than control, indicating a protective effect against GVHD when butyrate can bind FFA2/GPR43 (Fujiwara et al., 2018). Beside being a major source of energy for the intestinal epithelium (this allow energy-deprived cells to escape autophagy) and source of nutriment for microbes such as *Desulfotomaculum* spp. (Kuwahara, 2014), butyrate acts on the epigenetic regulation of genes by inhibiting an histone deacetylase (HDAC; Waldecker et al., 2008) and as an agonist for peroxisome proliferator-activated receptors (PPARs) that control both lipid metabolism and inflammation (Varga et al., 2011). Butyrate increases expression of proteins (such as junctional adhesion molecules/JAM/occludin) involved in the stability of tight junctions in colon epithelia; it regulates the neutrophil function and migration and inhibits inflammatory cytokine-induced expression of vascular cell adhesion molecule-1 (V-CAM1). It can pass through the enterocytes into the circulation. Butyrate and retinoic acid (RA) co-operate to regulate the innate immune response. Vitamin A is taken from food in the form of retinol, retinoic acid, or beta-carotene. In the gut, dendritic cells (DCs) metabolize vitamin A in RA, and RA co-operates with butyrate to induce mucosal-like $CD103^+$DCs differentiation required to trigger the differentiation and intestinal recruitment of $FoxP3^+$ T regulator (T reg) cells, IgA antibody secretion, and reduce inflammation (Qiang et al., 2017). The butyrate receptors, FFA2/GPR43 and FFA3/GPR41, are all found expressed on liver cells. At high

concentration, butyrate is expected to promote anti-tumor effect by optimizing the effector function of $CD8^+$ T cells that produce IFN-γ (Luu et al., 2018).

RADIOTHERAPY, ANTIBIOTICS/ CHEMOTHERAPY, AND OXIDATIVE STRESS-INDUCED LOSS OF CROSS FEEDING BACTERIA

Radiotherapy and antibiotic/chemotherapy treatments that are practiced to kill fast dividing cancer cells and as treatment of complications of allo-HCT are known to induce oxidative stress, where high levels of reactive oxygen species (ROS) and reactive nitrogen species (NOS) are generated. The free oxygen radicals hydroxyl radical (OH), superoxide anion (O_2-), hydrogen peroxide (H_2O_2) trigger the up-regulation of cyclooxygenases (COX), nitric oxide synthase, lipoxygenases, and nicotinamide adenine dinucleotide phosphate oxidase, leading to DNA damage, inflammation (radiation-induced enteritis/radiation-induced bowel injury), cell apoptosis, and also modify the microbiota homeostasis (Saha et al., 2017; Deleemans et al., 2019; Severyn et al., 2019). In a non-human primate model, ionizing radiations were also reported to induce up-regulation of tumor necrosis factor alpha (TNFα) and metalloprotease MMP7 (Zheng et al., 2015), likely affecting the intestinal epithelium barrier integrity and bacterial infiltration.

Radiation enteritis and dysbiosis were reported in patients who received a radiotherapy treatment. The dysbiosis was characterized by a relative higher abundance of Proteobacteria and Gammaproteobacteria and lower abundance of *Bacteroides*. A deeper analysis found an increase in oxidative stress resistant Enterobacteriaceae, Phyllobacteriaceae, and Beijerinckiaceae, whereas oxidative stress sensitive Bacteroidaceae and Ruminococcaceae were decreased (**Figure 3**; Wang et al., 2019). It confirms previous observation from radiation-induced intestinal chronic inflammation (Kumagai et al., 2018). Within the microbiota, some bacteria are resistant to oxidative stress (bacteria that synthesize the anti-oxidant enzyme superoxide dismutase, SOD, which neutralize O_{2-}, and catalase, and CAT, which neutralize H_2O_2), whereas others are highly sensitive. *Bifidobacterium* that are essential to fermentation are not able to produce butyrate although they are associated to a butyrogenic effect due to cross-feeding between *Bifidobacterium* and butyrate producing colon bacteria (Falony et al., 2009; De Vuyst and Leroy, 2011). *Bifidobacterium* are preferentially stimulated to growth in the presence of fructose oligosaccharides (FOS), inulin-type fructans (ITF), and xylo-oligosaccahride (XOS; Tuohy et al., 2001; Roberfroid, 2007; Lecerf et al., 2012). Cross-feeding between starch-degrading Bifidobacteria and lactate-converting, butyrate-producing colon bacteria (e.g., *Eubacterium hallii* and *Anaerostipes caccae*) has been demonstrated (Duncan et al., 2004). Indeed, *Eu. hallii* L2-7 and *A. caccae* L1-92 failed to grow on starch in pure culture, but in co-culture with *B. adolescentis* L2-32, butyrate was formed, indicating cross-feeding of metabolites to the lactate user bacteria (Belenguer et al., 2006). Obligate cross-feeding was also reported between *B. longum* BB53 and *A. caccae* DSM 14662 and *R. intestinalis* DSM 14610, *B. longum* acting as acetate producer for the butyrogenic bacteria (Falony et al., 2006), and in co-cultures of *B. adolescentis* L2-32 with *F. prausnitzii* S3/L3 or *F. prausnitzii* A2-165 with FOS as carbon source, resulting in acetate decrease and butyrate increase (Rios-Covian et al., 2015). Metabolic cross-feeding can occur *via* intercellular nanotubes among bacteria (Pande et al., 2015).

In the presence of carbohydrates, *Bifidobacterium* cross feed the butyrogenic bacteria that in turn produce butyrate (Rivière et al., 2016). However, *Bifidobacterium* strains such as *B. adolescentis* are highly sensitive to oxidative stress. It is therefore possible that during the chemotherapy that is practiced in patients with allo-HCT, such bacteria are lost. In methotrexate chemotherapy of lymphoblastic leukemia, a significant reduction of *Bifidobacterium*, *Lactobacillus*, and *Es. coli* was reported (Huang et al., 2012). Without cross-feeding, the production of butyrate will be drastically reduced. Treatment of patients with melatonin (*N*-acetyl-5-methoxytryptamine know to exhibit antioxidant activity) post-irradiation significantly increased both SOD and CAT, suggesting that the treatment restore the oxidative stress resistant bacteria (Musa et al., 2019).

Taken together, these results suggest that digestive bacteria sensitive to oxidative stress, capable of catabolizing lactose and increasing butyrate production are lost in GVHD. It was reported that the use of *Lactobacillus rhamnosus* (a butyric bacteria species) could prevent the occurrence of diarrhea in patients receiving radiotherapy (Delia et al., 2002). Other studies reported that use of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* were beneficial to the patients, yet, the results remain controversial (Kumagai et al., 2018; Wei et al., 2018), likely because these bacteria display phenotypic variation on strain level and each clone is different in terms of enzymatic equipment. More recently, it was reported that fecal microbiota transplant (FMT) improves the outcomes of an allo-HCT with GVHD (Severyn et al., 2019).

THE LACTASE PATHWAY

Recently, Stein-Thoeringer et al. (2019) reported that patients with allo-HCT who experienced GVHD were characterized by an increase in the lactose auxotroph (which require lactose for their *in vitro* growth) bacteria *En. faecium* in their gut microbiota that was accompanied by inflammation and intestine damages. It was hypothesized that LAB *En. faecium* may possibly mediate the pro-inflammatory process (**Figure 4**; Zitvogel and Kroemer, 2019).

The intestinal microbiota is the main source of lactase in adult. Bacterial lactase genes in the intestinal microbiota mainly derived from Actinobacteria, Proteobacteria, and Firmicutes (Long et al., 2018). It is excluded to draw general conclusions on a metabolism picture that would be common for all LAB. Each species and possibly each clone may behave differently depending on their/its enzymatic equipment. LAB are Gram-positive bacteria, acid-tolerant, low G+C content in the DNA, that belong to *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Lactococcus*, and *Oenococcus* genus have the capability to modify the environment in which they are delivered (Makarova et al., 2006). LAB genomes code between 1,700 and 2,500 proteins. The growth optimum for LAB is at pH

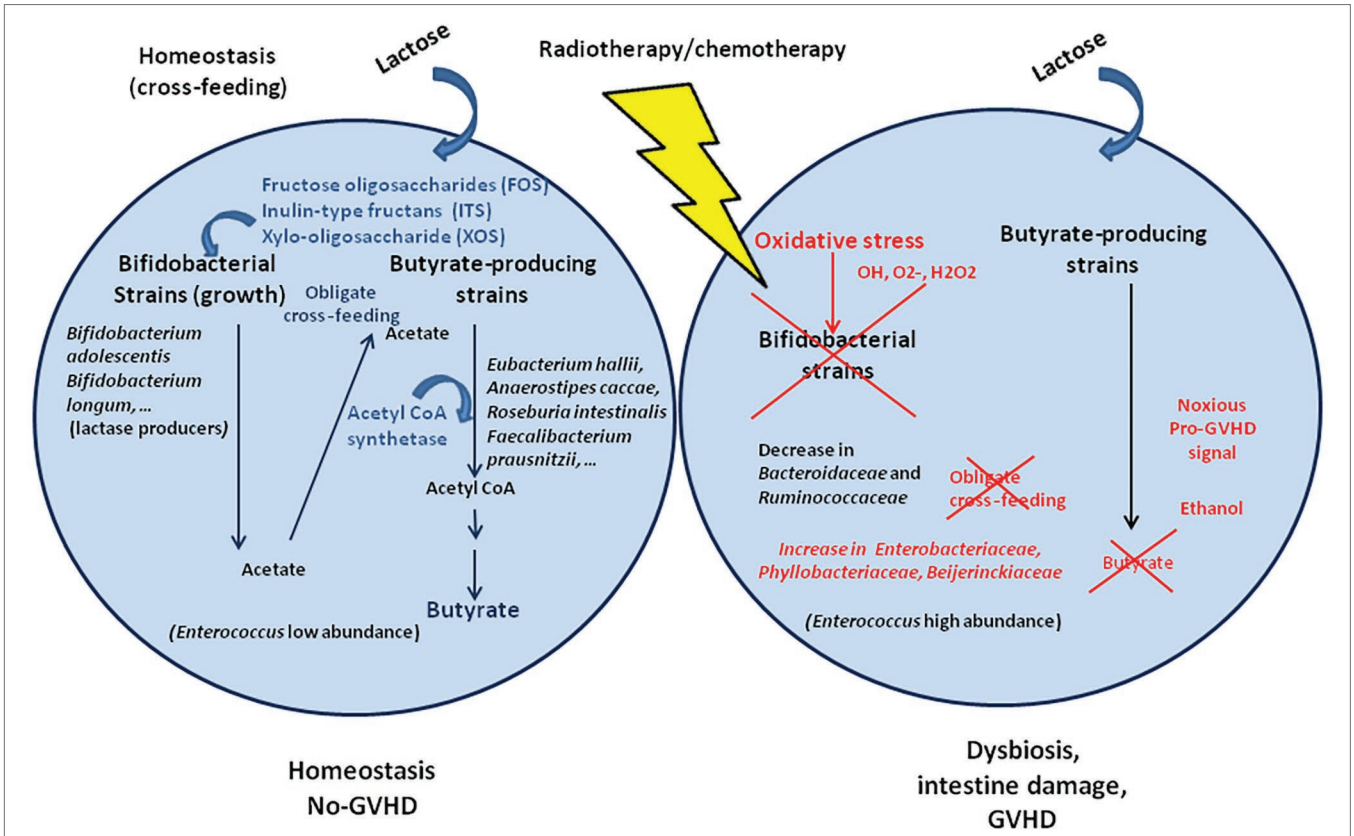

FIGURE 3 | Cross-feeding that take place between *Bifidobacterium* spp. and species of butyrate-producing colon bacteria. Within the microbiota, the bacteria equipped with superoxide dismutase and catalase anti-oxidant enzymes can be resistant to the free radical produced after radiotherapy/chemotherapy, whereas bacteria that lack these enzymes are highly sensitive to oxidative stress. *Bifidobacterium* that are essential to fermentation are not able to produce butyrate but produce acetate to cross-feed the butyrate producing colon bacteria. Under oxidative stress, the *Bifidobacterium* are killed and no longer play their role as cross-feeding bacteria with direct consequences on the production of butyrate by the butyrogenic bacteria.

5.5–5.8, and these bacteria have complex nutritional requirements for carbohydrates and other compounds such as fatty acids, peptides, vitamins, amino acids, peptides, nucleotide bases, and minerals. LAB produce lactase, an enzyme that catalyze the cleavage of carbohydrates (lactose, glucose, sucrose, or galactose) into lactic acid (Rhimi et al., 2009; Russo et al., 2017). Lactase is an important enzyme associated with lactose absorption. Dysbacteriosis associated to inhibition of intestinal lactase activity cause body diarrhea by affecting body's absorption of nutriments. A variety of diarrhea can be treated by supplementing oral intake with LAB or lactase (Luo et al., 2016).

Based on sugar fermentation patterns, LAB can be divided in two metabolic classes: first, the homofermentative bacteria that include some Lactobacilli (such as *Lactobacillus casei*, *Lactococcus lactis*, and *Lactobacillus plantarum*), most species of *Pediococcus* (such as *Pediococcus pentasaceus* and *Pediococcus acidilactici*), Enterococci (such as *En. faecium*), Streptococci, Tetragenococci, and Vagococci that ferment hexoses by the Embden-Meyerhof pathway. It was reported that *Streptococcus faecalis*, that produce glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, regulate the metabolism of glucose by the specific inhibitory interaction of the Embden-Meyerhof intermediate fructose-1,6-diphosphate with 6-phosphogluconate dehydrogenase (Brown and Wittenberger, 1971). In the presence of homofermentative LAB, lactate is the primary product with a production of two moles of lactate from one mole of glucose (homofermentative LAB are bacteria common to the dairy industry); second, the heterofermentative species include Leuconostocs, Oenococci, *Weissella* species, and some Lactobacilli (such as *Lactobacillus buchneri*). The heterofermentative LAB species produce one mole of lactate from one mole of glucose, as well as CO_2 and acetate and/or ethanol. High amount of ethanol was shown to be produced by *Lactobacillus fermentum* by glucose metabolism and *Weissella confusa* by fructose metabolism, whereas addition of pyruvate reduced their production of ethanol with a shift to acetate production (Elshaghabee et al., 2016). LAB can also synthesize different compounds such as bacteriocins (ribosomally synthesized anti-microbial peptides), H_2O_2, and enzymes capable to facilitate nutriment acquisition (such as protease, amylase,…), and other enzymes (such as β-galactosidase, galactose mutarotase, L-lactate dehydrogenase,…; Liao and Nyachoti, 2017). The apparent difference between the homofermentative and heterofermentative classes of LAB is the presence or absence of the fructose-1,6-diphosphate and/or phosphoketolases. Bacteria that produce xylose isomerase and xylulokinase can convert xylose, a pentose, to xylulose 5-phosphate.

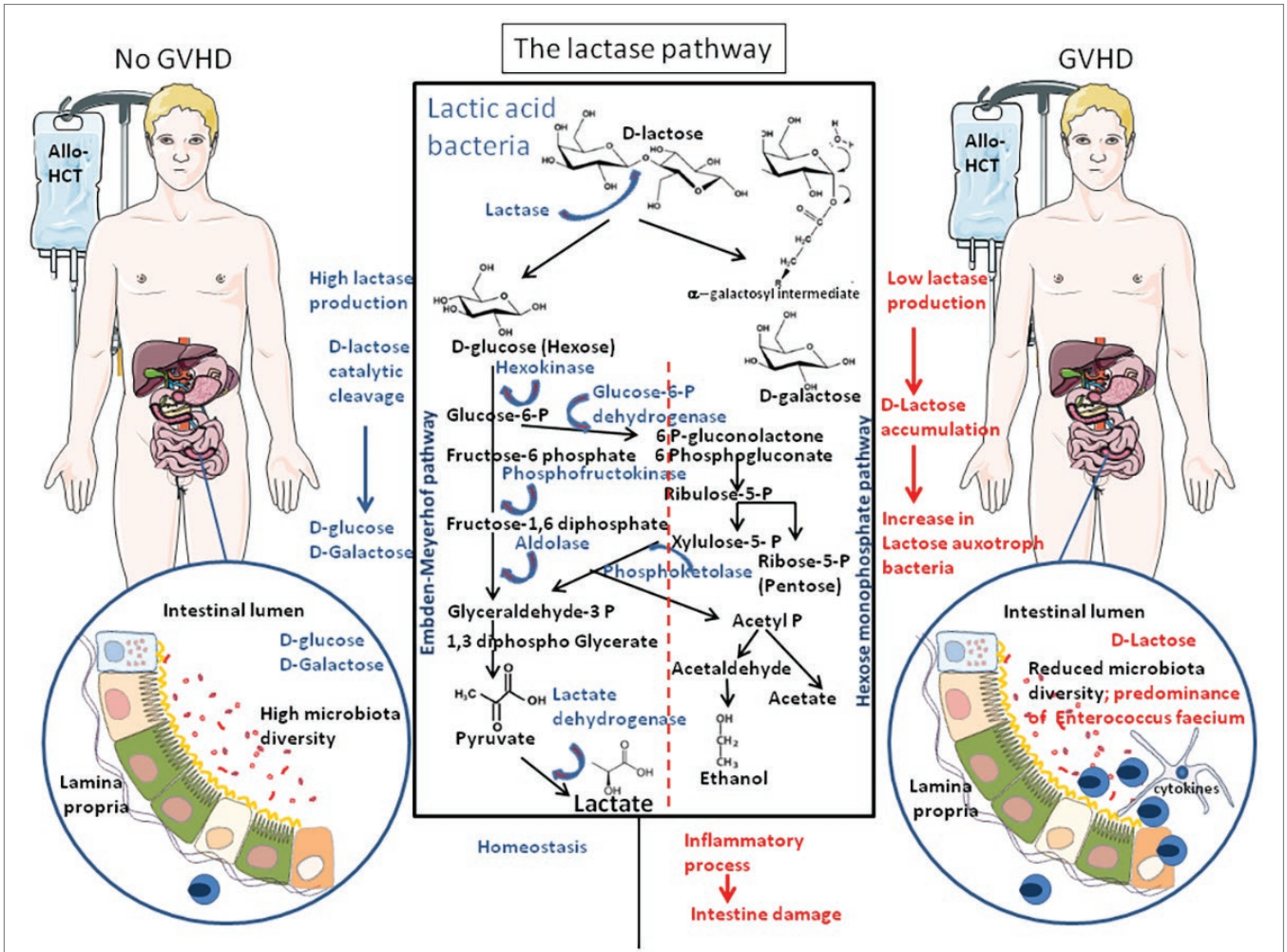

FIGURE 4 | The lactase pathway. The FGF21 was found to enhance MAPkinases (ERK1/2) phosphorylation in intestinal explants and to stimulate lactase production and glucose homeostasis. The lactic acid bacteria (LAB) of the intestinal microbiota are the main source of lactase in adult. Bacterial lactase genes in the intestinal microbiota derived from Actinobacteria, Proteobacteria, and Firmicutes. The bacterial species that transform the carbohydrates into lactate mainly belong to the genera *Bifidobacterium* and *Lactobacillus*, as well as *Streptococcus* and *Enterococcus*. Lactate is produced by reduction of pyruvate. LAB can be divided in two metabolic classes: first, the homofermentative bacteria that produce lactate includes some Lactobacilli and most species of *Pediococcus*, Vagococci, Streptococci, Tetragenococci, and Enterococci (including *Enterococcus faecium*), and the heterofermentative species include Leuconostocs, Oenococci, *Weissella* species, and some lactobacilli that produce lactate, carbon-dioxide (CO_2), acetate, and/or ethanol. Patients receiving allo-HCT as treatment of hematologic malignancies and who have an abundance of lactase-producing bacteria **(left)** produce lactate from lactose and are less sensitive to GVHD. Patients with allo-HCT, who carry a microbiota that produces low lactase, accumulate lactose that increases the abundance of lactose auxotroph bacteria (*En. faecium*) with a concomitant decrease of the microbiota diversity **(right)**, triggering pro-inflammatory responses, and serving as a breeding ground for GVHD.

In many bacteria, such as *Es. coli*, xylulose 5-phosphate is further catabolized to form glyceraldehyde-3 phosphate by the transketolase and transaldolase enzyme of the pentose phosphate pathway. Another xylose catabolic pathway used by heterofermentative bacteria such as *Clostridium acetobutylicum*, involves phosphoketolases that cleave xylulose 5-phosphate into acetyl-phosphate and glyceraldehyde-3-phosphate (Liu et al., 2012). In Bifidobacteria, phosphoketolases are key enzymes to convert fructose-6-phosphate to acetyl-phosphate and erythrose-4-phosphate (Grill et al., 1995). Recently, a *Collinsella aerofaciens* subspecies that uses butyric acid kinase and phosphatase butyryltransferase enzyme to metabolize sugars was described (Qin et al., 2019).

When produced at high concentration, lactate may in turn inhibit proline oxidase, thereby regulating the bioavailability of proline (a markedly reduced rate of proline degradation) in the liver (Kowaloff et al., 1977). A pathologic hyperprolinemia could be related to the hyperlactacidemia found in acquired lactic acidosis and alcoholic cirrhosis, and there are evidence in the literature that in infants with plasma concentration of lactate 8–10-fold normal, their plasma proline was two to three-fold the normal concentration and that patients with lactic acidosis showed a plasma concentration of proline five to six-fold the normal level. Since, proline can be a source for gluconeogenesis (a metabolic pathway that results in the generation of glucose from glucogenic amino acids, triglycerides,

glycerol, pyruvate, and lactate), increased lactate concentration in the liver may trigger acute hepatic gluconeogenesis that acts as metabolic fuels.

Several reports indicate that LAB induce adaptive immune responses (Kiczorowska et al., 2017; Han et al., 2018). Feeding piglets with *L. rhamnosus* prevented acute infectious diarrhea by triggering the lamina propria $CD3^+/CD4^+$ T cells activation (Zhu et al., 2014). *L. plantarum* was shown to stimulate anti-*Salmonella* immune response in pigs (Tran et al., 2016). In chickens, feed supplementation with *L. acidophilus* increases the production of $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cells in their gastrointestinal tract and peripheral blood (Asgari et al., 2016). LAB (mainly *Lactobacillus*) are currently considered as a possible probiotics and they are intensively studied to select the most valuable strains for commercial use (Aristimuño Ficoseco et al., 2018). Thus, Lactobacilli are mainly grouped in the growth promoter beneficial bacteria.

POSSIBLE RELATIONSHIP BETWEEN PATHOGENIC LACTIC ACID *ENTEROCOCCUS* GENUS AND GVHD

Although Enterococci are part of the normal intestinal microbiota, several species have a notable clinical implication, in particular *En. faecalis* and *En. faecium*. These Enterococci species cause a variety of diseases, including endocarditis, urinary tract infections, prostatitis, and cellulitis (Noris et al., 2010; Paganelli et al., 2016; Tien et al., 2017; Beganovic et al., 2018; Reissier et al., 2018). About 3% of the *En. faecalis* and *En. faecium* genomes encode enzymes involved in lactose and galactose metabolism (**Figure 5**; Stein-Thoeringer et al., 2019). *En. faecium*, is known for its ability to co-metabolize citrate and lactose and to produce high amounts of lactate (by conversion of pyruvate into lactate; Cabral et al., 2007). Risk factors for colonization and infection must be recognized, in particular prior treatment with antibiotics such as cephalosporins or quinolones. The veterinary use of vancomycin and avoparcin as growth factor in the feed supplementation of farm animals is likely one of the reasons for the selection of VanA strains that resist both vancomycin and avoparcin (a resistance that is uncommon except in Enterococci; Bortolaia et al., 2015). *En. faecalis* was reported as capable of producing extracellular superoxide and H_2O_2 that damage colonic epithelial cell DNA (Huycke et al., 2002). Unlike most commensal strains, the genome of multidrug-resistant (MDR) strains of *En. faecalis* clinical isolates are rich in mobile genetic elements and lack genome defense system composed by the clustered regularly interspaced short palindromic repeat (CRISP) and the CRISP-associated protein (CAS; Hullahalli et al., 2018). Moreover, *En. faecalis* incorporates to its membrane host-derived fatty acids found in human serum that protect the bacteria against membrane-damaging antibiotics (e.g., daptomycin; Saito et al., 2018). Another species of Enterococci, *Enterococcus gallinarum* was found to expand in mice treated with proton pump inhibitors (PPIs; Llorente et al., 2017). This strain is known to trigger pro-inflammatory pathways, to alter gut-barrier related molecules in the small intestine, and to translocate to the mesenteric lymph node, spleen and liver, inducing systemic autoimmunity both in mice and humans (Manfredo Vieira et al., 2018). *En. gallinarum* was also found to cause endocarditis (Angelos et al., 2018).

MICROBIOTA AND THE INNATE IMMUNITY: RELATIONSHIP TO GVHD

Interactions between the gut microbiota and the host immune system begin at birth and these two systems are involved in a complex interplay. The crosstalk that takes place determines the host immune inflammation status. Several studies reported that innate immune cells (e.g., neutrophils and inflammatory monocytes) are recruited to the gut shortly after allo-HCT (Schwab et al., 2014).

The gut microbiota shapes the gut mucosal immune system and the intestinal immunoglobulin A (IgA) produced by B cells predominantly target the commensal bacteria that reside in the small intestine, yet more IgA-producing B cells are recruited during inflammatory processes (Million et al., 2018). Gut homeostasis does not just rely on IgA production following microbial immune priming. Vitamin A derived RA together with butyrate were shown to maintain gut immune homeostasis through the induction of $CD103^+$ DC cells (in the mesenteric lymph nodes and colonic lamina propria) and recruitment of $FoxP3^+$ T reg cells (Qiang et al., 2017). The butyric acid bacteria *F. prausnitzii* (one of the most abundant Firmicutes in the gut) that are enzymatically equipped for the conversion of acetate into butyrate (it express acetate CoA transferase and butyrate kinase; Duncan et al., 2002), were found to exhibit anti-inflammatory properties through the stimulation of anti-inflammatory cytokine IL-10 expression and reduction of the pro-inflammatory cytokine IL-8 expression (Tremorali and Backhed, 2012; Heinken et al., 2014). Butyrate was also found to suppress expression of proinflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor (TNF)-α to reduce IL-12 expression, and butyrate-treated DCs showed a decreased capability in priming $CD4^+$ T cell proliferation. However, SFCAs at high concentration can also induce Th1 and Th17 cells upon immunological challenge and, therefore, also have the potential to induce inflammatory responses (production of IL-17 and IFNγ; Park et al., 2020). Moreover, the induction of the pro-inflammatory cytokine IL-23 in DCs by butyrate is linked to its role as a HDAC inhibitor for epigenetic modification of genes. Through binding to the retinoic acid-receptor -γt, IL-23 induces the production of the epithelial cell regenerative factor IL-22. Altogether, these observations indicate that $CD103^+$ DC cells and $FoxP3^+$ T reg cells, therefore, regulate the balance between gut tolerance and intestinal inflammation (**Figure 6**).

A high fat diet is known to alter the composition of the intestinal microbiota, notably decreasing the number of the Gram-positive Bifidobacteria bacteria and increasing the proportion of Gram-negative bacteria in the gut, and, hence, increase the release and plasma concentration of LPS and

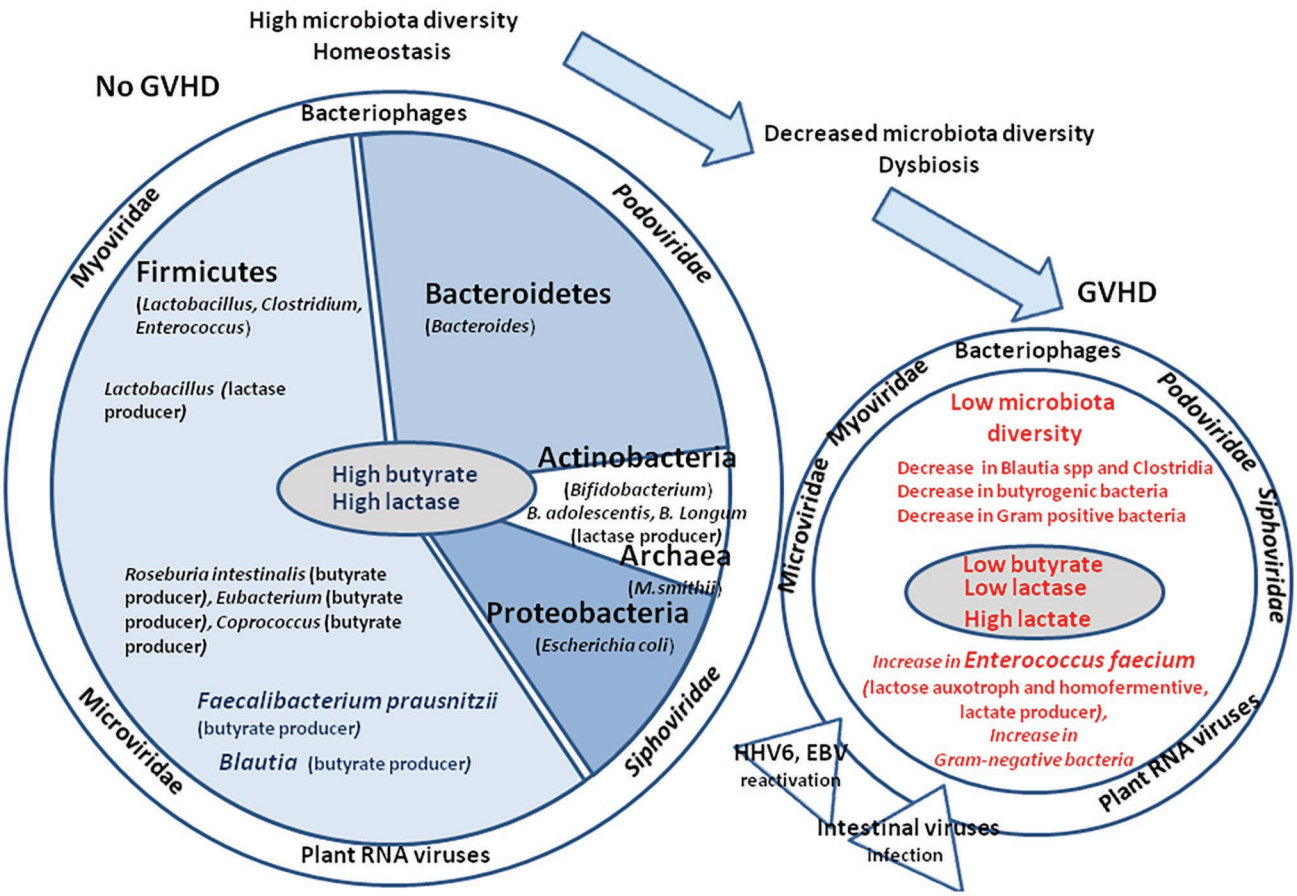

FIGURE 5 | Microbiome and virome in patients with allo-HCT. Many bacteria living in the intestinal tract **(left panel)** such as *Lactobacillus* sp., *Bifidobacterium* sp., *Bacillus* sp., and *Escherichia coli* produce lactase and play a major function in lactose absorption in the colon. Bacteria from the Firmicutes, Bacteroidetes, and Actinobacteria phyla play a role in bile acids metabolism and up-regulate mucosal defenses. *Faecalibacterium prausnitzii* is one of the rare bacteria that can convert acetate into butyrate, thereby contributing to the protection of the intestinal mucosa barrier and to the promotion of *Bifidobacterium* and *Lactobacillus* improving intestinal function. The presence of *F. prausnitzii* and *Blautia* spp. in the patient microbiota is a favorable prognosis mark for the patient. During GVHD **(right panel)**, an increase in the lactose auxotroph bacteria *En. faecium* is observed along with inflammation and intestine damages. A decrease in *Clostridium* spp. and *Blautia* spp. (butyrogenic bacteria) is accompanied by a significant reduction of fecal butyrate in GVHD. Gram-positive Lactobacilli, *Clostridia*, Bifidobacteria, and *Bacillus* spp. were less abundant in GVHD. In contrast, the proportion of Gram-negative bacteria (Enterobacteriaceae, Enterococci, and *Bacteroides/Prevotella* spp.) increased, as well as release of lipopolysaccharide (LPS) that trigger toll-like receptor (TLR) induction and low-grade chronic inflammation. During GVHD reactivation of viruses [e.g., human herpes virus 6 (HHV-6) or EBV] is frequently observed.

trigger TLR induction that generates low-grade chronic inflammation (Wagnerberger et al., 2012). Indeed, the TLR$^+$ villous microfold cells (M cells) present in the intestinal epithelium deliver luminal antigens to the underlying immune system either by transcytosis or microvesicle uptake, while alternatively M cell apoptosis and generation of transcellular pores through which DCs can gain access to the intestinal lumen may also allow capture of antigens and antigen presentation (Million et al., 2018). In mice model with deleted LPS receptors and CD14 mutants (CD14 is the co-receptor for the LPS-receptor TLR4), a hypersensitivity to insulin was reported, suggesting that high-fat diet-induced metabolic dysfunction occurs through the LPS/CD14 signaling pathway (Cani et al., 2007). LPS is a marker of Gram-negative bacteria death. High-fat diets have been shown to induce the passage of LPS from the intestine into the mesenteric lymphatic system (possibly through binding of chylomicrons, formed from dietary triglycerides, to LPS), leading to inflammation (Vreugdenhil et al., 2003). Production of chylomicrons was also reported capable to promote production the (TNF)-α proinflammatory cytokine. In mice models, cross linking of TLR9 by bacterial DNA stimulate GVHD whereas mutations in the *TLR9* and *TLR4* genes preventing bacterial LPS recognition were found to reduce GVHD (Calcaterra et al., 2008; Imado et al., 2010). Polymorphism of nucleotide-binding oligomerization domain 2 (*NOD2*) gene that encodes a receptor for the pathogen-associated molecular patterns (PAMPs) bacterial associated molecules is linked with a higher incidence of GVHD in HSC transplant recipients (Penack et al., 2010). The TLR5

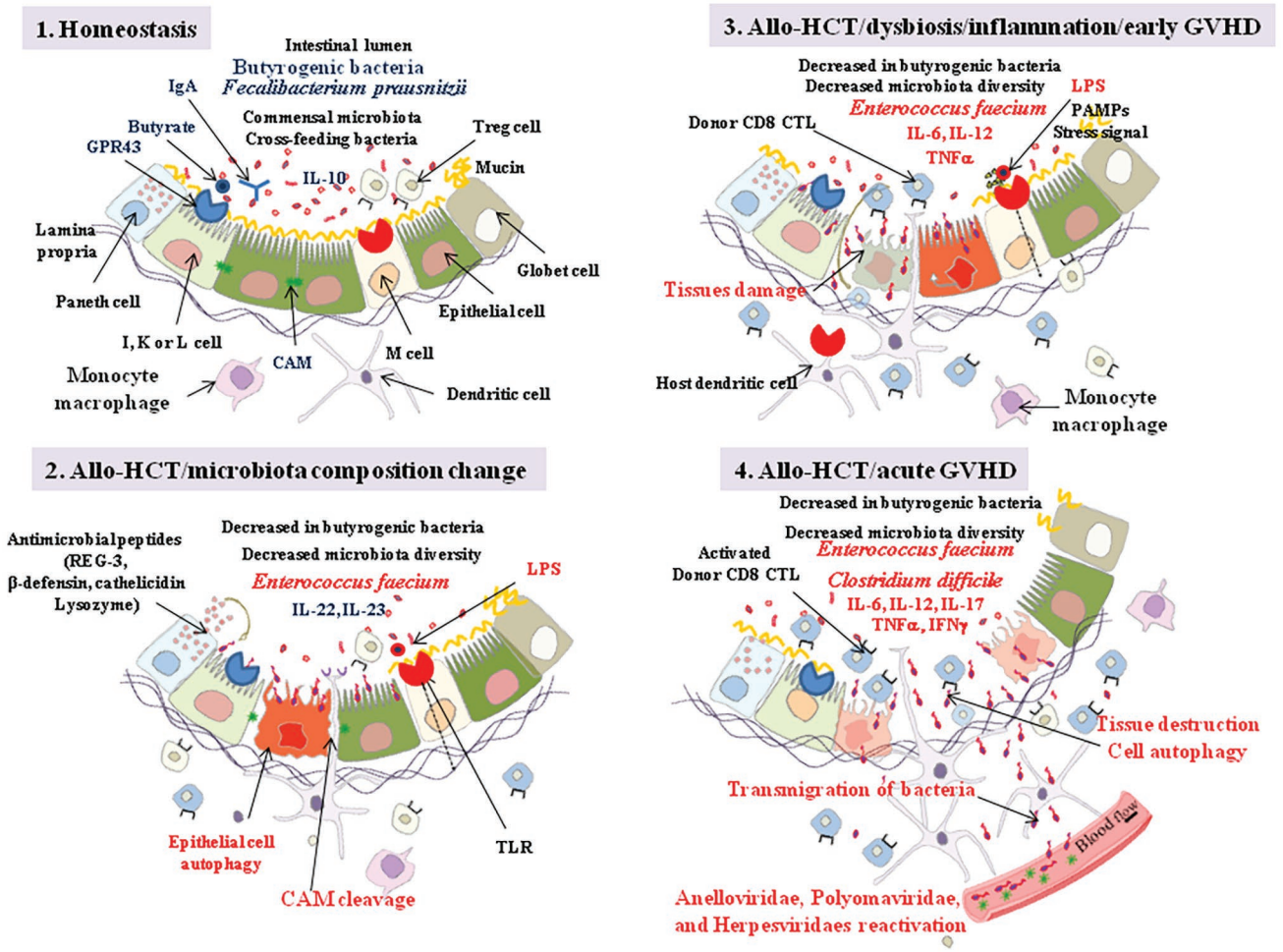

FIGURE 6 | Allo-HCT: from homeostasis to acute GVHD. Step 1: intestinal lumen microenvironment characteristic of homeostasis with high butyrate, presence of FoxP3 T reg cells, and anti-inflammatory interleukin 10 (IL-10). Butyrate controls the homeostasis through interaction with GPR43 expressed by enteroendocrine I, K, and/or L cells. The engagement of GPR43 triggers ERK signaling and subsequent NOD-LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome activation which promotes protection of intestinal epithelial cells and repairs by increasing IL-18 secretion. Butyrate also acts as a histone deacetylase inhibitor, thereby increasing expression of anti-apoptotic genes (e.g., BCL-B, a member of the BCL-2 family). The epithelial cells maintain a barrier through intercellular junctions that involve CAM, such as E-cadherin. Step 2: following allo-HCT, when the gut microbiota suffers quantitative and qualitative changes in bacteria composition (e.g., predominance of *En. faecium*), the release of bacterial LPS is considered as responsible for initiating an innate immune response through activation of TLRs at the surface of villous microfold cells (M cells). These cells deliver the luminal antigens to the underlying immune system to set up a whole arsenal of anti-bacterial innate immunity. Indole transitorily stimulates the production of IL-22 which protects the intestinal epithelial cells. Host DCs present recipient allo-antigens to donor $CD4^+$ and $CD8^+$ T cells that produce pro-inflammatory cytokines, serving as a breeding ground for GVHD. Through their MR1 molecules, dendritic cells (DCs) also activate host mucosal associated invariant T (MAIT) cells to secrete IL-17A, which enhances the intestinal barrier integrity. The Paneth cells produce antimicrobial proteins (e.g., C-type lectin REG3γ, β-defensins, cathelicidins, and lysozyme). Sheddases produced by bacteria or by eukaryotic cells through bacterial signaling initiate the cleavage of cell adhesion molecule (CAM; e.g., cleavage of E-cadherin), creating damages in the intestinal barrier. Step 3: the sequential detection of stress signals, tissue damages, and pathogen-associated molecular patterns (PAMPs; e.g., lipopolysaccharide, flagellin, peptidoglycan, lipoproteins, and unique bacterial nucleic acid structures) by the host antigen presenting cells activates the immune system. $KLRG1^+$ dendritic cells and monocytes/macrophages, $CD103^+$ T-cells, $KLRG1^+$ T-cells, and other immune cell subpopulations, colonize the lamina propria. To challenge the dysbiosis, epithelial cell autophagic clearance and dead cells renewal accelerates but remains unsuccessful due to aggravation of the pro-inflammatory process that combine activation of anti-recipient host alloantigens donor $CD8^+$ cytotoxic T cells (CTL) and proinflammatory cytokines (IL-6, IL-12, TNFα) production. Alloreactive T cells from the donor attack healthy tissues in the recipient allo-HCT patient. Step 4: the inflammatory process develop accompanied with transmigration of bacteria outside the intestinal lumen (*Clostridium difficile* has been frequently found associated with severe GVHD), reactivation of viruses, further tissue destruction and global aggravation (spread to other tissues) of the disease with characteristic symptoms including abdominal cramping and diarrhea, and biological markers of abnormal liver function (elevation of alkaline phosphatase and bilirubin). GVHD can be also monitored by skin, liver, and gut biopsis. GVHD is also associated with lower urine levels of 3-indoxyl sulfate.

agonist fagellin extracted from bacterial flagella was shown to reduce GVHD and preserve long post-transplant immune reconstitution characterized by more $Fop\text{-}3^+$ T regulator (T reg) cells (Hossain et al., 2011). Studying the role for TLR9 and its downstream signaling adaptor MYD88 in an intestine GVHD model, Heimessat et al. (2010) observed a shift towards Enterobacteriaceae, Enterococci, and *Bacteroides*/*Prevotella* spp. being elevated in the colon lumen during GVHD, whereas

Lactobacilli, *Clostridia*, Bifidobacteria and *Bacillus* spp. were less abundant. *In fine*, GVHD is mediated by $CD8^+$ CTLs that express anti-host specificities. In a murine model, it was found that GVHD may be inhibited by preventing $CD8^+$ CTL migration into the Peyer's patch either by disrupting the gene encoding the CCR5 chemokine receptor or by blocking the integrin α4β7-mucosal vascular addressin (MAdCAM-1) interaction into the gut Peyer's patches (Murai et al., 2003). Since mutants in the integrin α4β7 can also affect the integrin binding to E-cadherin (Higgins et al., 2000), it remains possible that such mutants might also affect the anti-bacterial CTL immune response through aberrant homing signals that involve E-cadherin, soluble E-cadherin, CD103, and KLRG1, as previously suggested (Devaux et al., 2019).

IS THERE ROOM FOR VIRUSES IN GVHD?

Immense populations of viruses, among which bacteriophages, are present in the human gut, and lysogenic or temperate phages are able to integrate their chromosome into the bacterial genome, sometimes altering the phenotype of host bacteria. A number of different eukaryotic viruses have also been found in the human gut virome with a predominance of the pepper mild mottle virus, a plant-infecting RNA virus derived from diet (Zhang et al., 2006). A very elegant study by Minot et al. (2011) reported the presence of *Myoviridae*, *Siphoviridae*, *Podoviridae*, *Tectiviridae*, *Inoviridae*, and *Microviridae* in human stools from healthy donors. Among double stranded DNA viruses found in the human gut, a predominance of *Podoviridae* followed by *Siphoviridae* and *Myoviridae* was reported in healthy humans while *Microviridae* dominated the single strand DNA viruses (Kim et al., 2011). Kim et al. (2011) also reported the rare presence of picornaviruses, herpesviruses, and poxviruses. Many viruses (e.g., rotaviruses, caliciviruses, astroviruses, enteric adenoviruses, toroviruses, and parechoviruses) are known to induce gastroenteritis in humans, the leading cause of diarrheal disease being rotaviruses, a *Reoviridae* (Clark and McKendrick, 2004). Therefore, it cannot be ignored that the virome could influence GVHD just as easily as the microbiome. Although eukaryotic cell-borne viruses are minor components of the human gut virome compared to the bacteriophages, latent viral infections can be reactivated in patients with allo-HCT who experience a phase of immunosuppression. Norovirus induced gastroenteritis was found to be a major threat to patients to allo-HCT (Schwartz et al., 2011). Reactivation of human herpes virus 6 (HHV-6) was reported to be a predictive factor for acute GVHD (Pichereau et al., 2012). The HHV-6 reactivation reported in 35% of patients who received an allo-HCT (sometimes associated with a subsequent cytomegalovirus reactivation) was found predictive of mortality after allo-HCT (Zerr et al., 2012). Feghoul et al. (2015) reported that human adenovirus infections constitute a major cause of morbidity in pediatric allo-HCT patients. The incidence rate at day 100 was 35.9% for the adenovirus digestive infections and 24.0% for the systemic infections. Infection with herpes simplex virus-1 and 2 is mostly seen during the pre-engraftment phase, whereas cytomegalovirus and HHV-6 are mainly found during the post-engraftment phase, and Epstein-Barr virus and varicella-zoster virus infections are often observed after the 100th day post-transplant (Sahin et al., 2016).

Yang et al. (2016) reported unexpected results indicating that mice treated with antiviral cocktail display more severe dextran sulfate sodium-induced colitis than untreated mice suggesting that gut resident viruses may insure maintenance of gut homeostasis. DCs isolated from colon of inflamed mice produced interferon-β in a TLR-dependent manner. When mice were reconstituted with toll-like receptors or rotavirus, colitis symptoms were significantly ameliorated, suggesting that enteric rotaviruses damped gut inflammation *via* toll-like receptors TLR-3 and TLR-7-mediated interferon β production. Consequently, it could not be excluded that human gut eukaryotic viruses can also be beneficial to patients in limiting GVHD. Legoff et al. (2017) reported that within a cohort of patients who received allo-HCT, patients who experienced enteric GVHD had both decreased richness of virome and higher abundance of *Microviridae*. *Anelloviridae*, *Polyomaviridae*, and *Herpesviridae* were found in immunocompromised allo-HCT patients likely related to viral reactivation. Recently, it was reported that myxoma virus, a *Poxviridae* that exhibits oncolytic activity against various hematologic malignancies like multiple myeloma or acute myeloid leukemia could be used, has a tool for *ex-vivo* treatment of allo-HCT with evidence of possible GVHD abrogation without impairing graft-versus-tumor effects against residual cancer cells (Villa and McFadden, 2018).

DISCUSSION

Allo-HCT is a potentially curative treatment of hematologic malignancies. However, the effectiveness of this treatment remains limited by the high incidence of acute GVHD, which is the principal cause of death in allo-HCT (Riwes and Reddy, 2018). Pioneers' experimental studies carried out in the 1970s already indicated less acute GVHD in mice which received allo-HCT in germ-free conditions or experimental group receiving gut decontamination antibiotics (van Bekkum et al., 1974). The influence of intestinal bacteria on the development of acute GVHD was further confirmed in patients receiving allo-HCT as treatment of hematologic malignancies. More than 20 years ago, evidence was obtained that antimicrobial chemotherapy (metronidazole and ciprofloxacin) targeted to intestinal anaerobic bacteria in marrow transplant recipients significantly reduced the severity of acute GVHD (Beelen et al., 1999). Although it was hypothesized that allogenic GVHD is driven by initial interaction between APCs that encounter donor T lymphocytes that in turn release pro-inflammatory cytokines recruiting allo-reactive T cells, the molecular crosstalk that account for allo-HCT tolerance or GVHD was largely ignored (Magenau et al., 2016). Indeed, it is very likely that during the set up of post-transplant GVHD, the control of

the innate immune response is dependent on both the bacteria which produce butyrate and those which produce lactase. The precise quantitative and qualitative adjustment of these bacterial populations is probably the key to the balance between immune tolerance and inflammatory process/GVHD.

A decrease in SCFAs (butyrate) was found to be associated with an increased mortality from GVHD following allo-HCT transplantation (Mathewson et al., 2016). Next, the same research group reported that the GVHD protective effect of SCFAs requires GPR43-mediated intracellular signaling that triggers ERK phosphorylation, ERK-dependent activation of IL-18, and activation of the NOD-LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome (Fujiwara et al., 2018). GPR43 is expressed by intestinal epithelial cells, as well as by antigen presenting cells (APCs, such as macrophages and DCs). Fujiwara et al. (2018), reported that macrophages and DCs isolated from ileum and colon of allo-HCT recipients show greater expression of GPR43 (whereas *in vivo* GPR43 expression on donor T cells is weak), but only the expression of GPR43 on non-hematopoietic cells (presumably intestinal epithelial cells) was involved in protective effect of SCFAs against GVHD. Moreover, a diet supplemented with butyrate mitigates GVHD. According to Golob et al. (2019), the beneficial response to butyrate would be biphasic with the first phase corresponding to the prevention of acute GVHD and the second phase dealing with the recovery, once colitis is durably established. It was also reported that the abundance of *Clostridia* decreased in the microbiota of allo-transplant patients that experienced GHVD and was accompanied by alteration in gastrointestinal microbiota-derived butyrate (Mathewson et al., 2016). The central role played by butyrate in the prevention of GVHD indicates that the presence of butyrogenic bacteria is essential for GVHD prevention. Butyrate is the main microbiota-derived regulator of gut mucosal immunity. Butyrogenic bacteria are considered oxidative stress sensitive. Most of these bacteria do not synthesize butyrate in the absence of cross-feeding bacteria that are oxidative stress sensitive. Under oxidative stress, the presence of butyrogenic bacteria in the microbiota decrease and oxidative stress-resistant strains (that encode enzymes such as catalase or superoxide dismutase required to deal with oxidative stress) are selected and trigger secretion of pro-inflammatory cytokines by immune response cells (Million et al., 2018).

In human, lactase persistence is associated with enhanced milk intake, higher waist circumference, and cardiometabolic abnormalities. Higher dairy intake was associated with higher body mass index supporting a causal link between lactose ingestion and body weight (Huang et al., 2018). When consuming dairy products, a person with lactase intolerance (the most common cause of the disease being a genetic mutation in the promoter of the gene that codes for lactase resulting in loss of intestinal lactase) may experience the symptoms of lactose intolerance which includes nausea, abdominal pain, gas, bloating, and diarrhea (Misselwitz et al., 2019; Kastl et al., 2020). Beside lactose, dairy products also provide numerous biologically active factors including growth factors, including the fibroblast growth factor FGF21 (mainly produced in the liver, brown and white adipocyte tissues, and pancreas) that was found to enhance MAPkinases (ERK1/2) phosphorylation in intestinal explants and to stimulate lactase production and lactose absorption (Gavaldà-Navarro et al., 2015). Gavaldà-Navarro et al. (2015) reported that mice feed with milk from FGF21 knockout mice showed decreased expression of lactase and maltase glucoamylase in the ileum. FGF21 binds to the FGFreceptor in complex with βKlotho to trigger cell signaling (Kurosu et al., 2007), yet the mode of FGF21 regulation of lactase expression by intestinal cells remains to be explored. This growth factor is known for stimulating the oxidation of fatty-acids, the production of ketone bodies, and inhibition of lipogenesis, thus regulating the glucose-lipid metabolism (Tezze et al., 2019). Recently, it was reported that feeding allo-transplanted mice with lactose free diet mitigated GVHD and reduced post-transplant *Enterococcus* proliferation (the optimal growth of which depends on lactose availability), whereas in mice feed with a dairy products diet, the lactase expression declined in the duodenum during the course of transplantation and induced a pathological state resembling lactose intolerance (Stein-Thoeringer et al., 2019).

Holler et al. (2014) reported a shift toward Enterococci after allo-HCT with a decrease in the obligate Firmicutes anaerobic bacteria that was much pronounced in the patients treated with antibiotics. Stein-Thoeringer et al. (2019) hypothesized that intestinal mucosal damages caused by irradiation or allo-reactive T cells may reduce the production of lactase from the small intestine enterocytes, that duodenal lactase progressively decline allowing undigested lactose to reach the lower intestinal tract where it serves as metabolic fuels for the growth of *Enterococcus* (e.g., *En. faecium* in human, *En. faecalis* in mice). Nalle et al. (2019) recently reported that MLCK210-deficient mice exhibited limited GVHD and were protected from epithelial barrier damages and $CD8^+$ T cells proliferation. These authors discriminate the initiation of GVHD considered to be tight junction-independent from GVHD propagation that is MLCK210-dependent. Stein-Thoeringer et al. (2019) reported that *En. faecium* (only recently considered a human pathogen) dominate in patients with allo-HCT between 3-week and 3-month post-transplantation and that fecal domination by *Enterococcus* in the early post-transplant period (the first 3-week post-transplant) was associated with increased GVHD and overall mortality. They also found the VanA operon in a subset (37.4%) of patients. In an animal model, Stein-Thoeringer et al. (2019) reported a transient expansion of *En. faecalis* in GVHD mice and administration of *En. faecalis* aggravated the GVHD, thus demonstrating a direct link between the *Enterococcus* predominance and the GVHD. A major breakthrough came along with the attempt to demonstrate that the post-transplant defect of mucosal defense mechanisms facilitate the expansion of *Enterococcus*. They found that intestinal antimicrobial peptides of the Reg3 family, known to suppress the growth of vancomycin resistant Enterococci (VRE bacteria) that exploit innate immune deficits (Brandl et al., 2008), were reduced in the ileum of GVHD mice. Obviously, the primary strategy to prevent GVHD after allo-HCT is immunosuppression, but such treatment may

increase the risk of enteropathogenic bacteria invasion. Stein-Thoeringer et al. (2019) also provided an elegant demonstration that *En. faecium*-dominated microbiota observed after HCT were enriched in bioactive compounds involved in lactose and galactose degradation pathway (with similar result in *En. faecalis*-dominated microbiota in mice). *En. faecalis* expresses the gelatinase (GelE) a matrix metalloprotease, so-called sheddase, that cleaves E-cadherin (Steck et al., 2011) and activates the protease-activated receptor 2 (PARP2), a transmembrane G-protein-coupled receptor (Maharshak et al., 2015). These molecules contribute to the disruption of intestinal barriers and inflammation. According to the UniProt database[1], *En. faecalis* also encodes a predicted htrA protein serine protease, another candidate sheddase (Devaux et al., 2019).

In mice animal model, it was observed that PPIs (namely omeprazole) administered to mice cause reduced gastric secretion and favor the expansion of *Enterococcus* species including *En. faecalis* (Llorente et al., 2017). This favor bacterial translocation in mesenteric lymph nodes and the liver with subsequent inflammation, hepatocytes death, and non-alcoholic steatohepatosis. Mice that lack expression of the TLR2 (a cell membrane receptor that recognizes peptidoglycan from Gram-positive bacteria) or the myeloid differentiation primary response 88, MYD88 (intracellular adaptor molecule for TLRs), lack innate immune response and were protected from *En. faecalis*-induced inflammation, steatosis, and liver injury. *En. faecalis* was found capable of suppressing the innate immune response of macrophages through repression of NF-κB signaling (Tien et al., 2017). In a human cohort, administration of omeprazole for preclinical evaluation, the frequency of *Enterococcus* in the fecal samples was increased after 2-week of PPI treatment. This corroborates previous observation indicating that an omeprazole therapy in cirrhosis demonstrated a shift in fecal microbiota composition (Bajaj et al., 2014; Merli et al., 2015). Innate immune response that follows the engagement of TLRs is involved in the process of GVHD. The TLR5 agonist fagellin was shown to reduce GVHD and preserve long post-transplant immune reconstitution characterized by more Fop-3$^+$ T regulator (T reg) cells (Hossain et al., 2011). Although it remains to be documented in humans suffering from ten-eleven translocation (TET) methylcytosine dioxygenase 2 (*TET2*)-deficiency, in an animal model it was reported that mutations in *TET2*, which encodes an epigenetic modifier enzyme that influence the activation of regulatory CD4$^+$ T cells (T reg cells) *via* FoxP3 (Yue et al., 2016), can be associated with dysfunction of the small intestine barrier, bacterial translocation and IL-6 production known to be a critical activator of myelopoiesis in response to systemic bacterial dissemination (Meisel et al., 2018).

Clostridium difficile has also been implicated as a potential trigger of immune reaction that can contribute to the development of GVHD. Working with a cohort of 75 allo-HCT patients, Chakarbati et al. (2000), found that *C. difficile* was frequently associated with severe GVHD (grade 3–4) and the presence of the bacteria in stool worsened the pathophysiology of GVHD in 60% of patients. The relationship between the presence of *C. difficile* and GVHD was also reported by Dubberke et al. (2007) who reported that patients with *C. difficile* showed a higher propensity to develop new-onset GVHD and severe forms of GVHD. Similar observations were reported from the investigation of a cohort of 822 allo-HCT patients regarding the risk to develop severe GVHD at day 60 and day 100 after allo-HCT when *C. difficile* was present in the patients (Trifilio et al., 2012). The incidence was more than 20% in patients who were older than 60 years and carried a vancomycin-resistant (VRE) *C. difficile*. Indeed, *C. difficile* positive diagnosis was found to precede GVHD diagnosis in 85.7% of allo-HCT patients who developed gut GVHD while the overall 1-year incidence of *C. difficile* was 9.2% among a cohort of 999 allo-HCT patients (Alonso et al., 2012; Alonso and Marr, 2013). Although the presence of VRE *C. difficile* was not investigated in the main papers discussed in this review, it can be hypothesized that the decreased microbiota diversity (butyrogenic bacteria) facilitates the clonal expansion *C. difficile* strains in the gut of these patients (Hocquart et al., 2018), which in turn represents an aggravating factor regarding the risk of mortality by GVHD of allo-HCT patients.

CTLA-4, currently known as a target for immune checkpoint inhibitor antibodies, was originally described as a T cell surface molecule that negatively influence immune response by competing with CD28 for binding to ligands on APCs. In patients with severe alcoholic hepatitis, it was reported that PD-1 immune checkpoint-receptor inhibition restored the adaptive antibacterial T cell response which otherwise was defective (Markwick et al., 2015). The approval of immune checkpoint inhibitors (such as CTLA-4 or PD1/PD-L1 inhibitor antibodies) in the treatment of cancers has changed the outcome of several of such severe diseases and highlighted the strong molecular crosstalk between the microbiota and the immune system (Zitvogel et al., 2018; Gong et al., 2019). Defects in the microbiota can compromise the therapeutic efficacy or have secondary side effect on the gut. In melanoma therapy, anti-PD1 and anti-CTLA-4 antibodies have been approved. However, immune-mediated colitis in patients with melanoma after dual checkpoint inhibitors treatment was described (Thalambedu et al., 2019). A variety of genera can reduce the immune checkpoint inhibitor-induced colitis possibly by limiting the inflammation through expansion of CD4$^+$/FoxP3$^+$ T reg cells and/or production of anti-inflammatory cytokines (Dubin et al., 2016). In a murine model of melanoma, CD8$^+$ T cell activation in response to PD-L1 inhibitor correlated with mice, which received fecal transplantations from patients abundant in bacteria from the Ruminococcaceae family and *Faecalibacterium* spp., whereas nonresponders were characterized by elevated presence of CD4$^+$/FoxP3$^+$ T reg cells and received stools which were abundant in Bacteroidales (Gopalakrishnan et al., 2018).

The experience accumulated in the field of cancer therapy with immune checkpoint inhibitors provides evidence that the microbiota govern the balance between CD8$^+$ T cell activation and inflammation on one side and CD4$^+$/FoxP3$^+$ T reg cells and lack of inflammation on the other side. It can be extrapolated that high butyrate allows CD4$^+$/FoxP3$^+$ T reg cells expansion and prevents inflammation, and GHVD reaction whereas low lactase induces lactic acid bacteria expansion, CD8$^+$ T cell activation, inflammation and GVHD. However, we are just

[1]https://www.uniprot.org/uniprot/Q82ZM6

beginning to understand what could be the role of the microbiota and the impact of cross feeding in GVHD. First, variation in the composition of the microbiota between allo-HCT patients that lack GVHD and those who unfortunately experience GVHD is currently well established. Second, the role played by butyrogenic and lactic acid bacteria begun to be understood. However, it is now necessary to enter more deeply into the characterization of the bacterial species that distinguish homeostasis from dysbiosis in non-GVHD versus GVHD patients and to study their enzymatic equipment which can vary both at the level of the bacterial strain and at the clone level. There is currently evidence that the adverse outcomes of irradiation and antibiotics/chemotherapy treatments (oxidative stress) on gut microbiota could be avoided by using probiotics and/or fecal microbiota transplant, opening new perspective for the prevention of GVHD in allo-HCT patients (Delia et al., 2002; Kaito et al., 2018; Severyn et al., 2019; Shouval et al., 2019). The next challenge is to offer transplant patients a beneficial combination of easy-to-consume probiotics, which should prevent the occurrence of GVHD. Strict anaerobic bacteria are not the easiest to cultivate and produce in large quantity for therapeutic application. We have recently reported that oxygen-sensitive bacteria can maintain butyrate production despite the presence of oxygen when treated with antioxidants (Million et al., 2020). Among lactic acid bacteria, the *Bifidobacterium* seem to be the best candidates as anti-GVHD probiotics. In contrast, the Enterococci which also metabolize lactose are suspected of being deleterious and responsible for the production of noxious substances. If this therapeutic cocktail must contain cross feeding bacteria and butyrogenic bacteria, it will be necessary to exclude those which are known for their deleterious side effects (i.e., *Clostridium butyricum*, associated with enterocolitis, or *Mediterraneibacter gnavus*, associated with obesity) and to investigate the beneficial cooperative effects of bacteria such as *B. adolescentis*, *B. longum*, *Eu. hallii*, *R. intestinalis*, and particularly *F. prausnitzii*.

AUTHOR CONTRIBUTIONS

CD, MM, and DR contributed to the conception of the manuscript. CD wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Alonso, C. D., and Marr, K. A. (2013). *Clostridium difficile* infection among hematopoietic stem cell transplant recipients: beyond colitis. *Curr. Opin. Infect. Dis.* 26, 326–331. doi: 10.1097/QCO.0b013e3283630c4c

Alonso, C. D., Treadway, S. B., Hanna, D. B., Huff, C. A., Neofytos, D., Carroll, K. C., et al. (2012). Epidemiology and outcomes of *Clostridium difficile* infections in hematopoietic stem cell transplant recipients. *Clin. Infect. Dis.* 54, 1053–1063. doi: 10.1093/cid/cir1035

Ames, N. J., Sulima, P., Ngo, T., Barb, J., Munson, P. J., Paster, B. J., et al. (2019). A characterization of the oral microbiome in allogeneic stem cell transplant patients. *PLoS One* 7:e47628. doi: 10.1371/journal.pone.0047628

Andermann, T., Peled, J., Ho, C., Reddy, P., Riches, M., Storb, R., et al. (2018). The microbiome and hematopoietic cell transplantation: past, present, and future. *Biol. Blood Marrow Transplant.* 24, 1322–1340. doi: 10.1016/j.bbmt.2018.02.009

Angelos, T., Nikolaos, S., Nikolaos, K., Nektarios, P., and Evdokia, P. (2018). A rare case of tricuspid valve endocarditis caused by *Enterococcus gallinarum* in a patient with recurrent urinary tract infection. *Cardiovasc. Hematol. Disord. Drug Targets* 18, 162–164. doi: 10.2174/1871529X18666180302113428

Aristimuño Ficoseco, C., Mansilla, F. I., Maldonado, N. C., Miranda, H., Nader-Macias, M. E. F., and Vignolo, G. M. (2018). Safety and growth optimization of lactic acid bacteria isolated from feedlot cattle for probiotic formula design. *Front. Microbiol.* 9:2220. doi: 10.3389/fmicb.2018.02220

Arrieta, M. C., Stiemsma, L. T., Amenyogbe, N., Brown, E. M., and Finlay, B. (2014). The intestinal microbiome in early life: health and disease. *Front. Immunol.* 5:427. doi: 10.3389/fimmu.2014.00427

Asgari, F., Madjid, Z., Falak, R., Bahar, M. A., Nasrabadi, M. H., Raiani, M., et al. (2016). Probiotic feeding affects T cell populations in blood and lymphoid organs in chickens? *Benef. Microbes* 7, 669–675. doi: 10.3920/BM2016.0014

Bajaj, J. S., Cox, I. J., Betrapally, N. S., Heuman, D. M., Schubert, M. I., Ratneswaran, M., et al. (2014). System biology analysis of omeprazole therapy in cirrhosis demonstrates significant shift in gut microbiota composition and function. *Am. J. Physiol. Gastrointest. Liver Physiol.* 307, G951–G957. doi: 10.1152/ajpgi.00268.2014

Bansal, T., Alaniz, R. C., Wood, T. K., and Jayaraman, A. (2010). The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. *Proc. Natl. Acad. Sci. U. S. A.* 107, 228–233. doi: 10.1073/pnas.0906112107

Basson, M. D., Liu, Y. W., Hanly, A. M., Emenaker, N. J., Shenoy, S. G., and Gould Rothberg, B. E. (2000). Identification and comparative analysis of human colonocyte short-chain fatty acid response genes. *J. Gastrointest. Surg.* 4, 501–512. doi: 10.1016/S1091-255X(00)80093-1

Beelen, D. W., Elmaagacli, A., Müller, K. D., Hirche, H., and Schaefer, U. W. (1999). Influence of intestinal bacterial decontamination using metronidazole and ciprofloxacin or ciprofloxacin alone on the development of acute graft-versus-host disease after marrow transplantation in patients with hematologic malignancies: final results and long-term follow-up of an open-label prospective randomized trial. *Blood* 93, 3267–3275.

Beganovic, M., Luther, M. K., Rice, L. B., Arias, C. A., Rybak, M. J., and LaPlante, K. L. (2018). A review of combination antimicrobial therapy for *Enterococcus faecalis* bloodstream infections and infective endocarditis. *Clin. Infect. Dis.* 67, 303–309. doi: 10.1093/cid/ciy064

Belenguer, A., Duncan, S. H., Calder, G., Holtrop, G., Louis, P., Lobley, G. E., et al. (2006). Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. *Appl. Environ. Microbiol.* 72, 3593–3599. doi: 10.1128/AEM.72.5.3593-3599.2006

Blazar, B. R., Murphy, W. J., and Abedi, M. (2012). Advances in graft-versus-host disease biology and therapy. *Nat. Rev. Immunol.* 12, 443–458. doi: 10.1038/nri3212

Booijink, C. C., El-Aidy, S., Rajilic-Stojanovic, M., Heilig, H. G., Troost, F. J., Smidt, H., et al. (2010). High temporal and inter-individual variation detected in the human ileal microbiota. *Environ. Microbiol.* 12, 3213–3227. doi: 10.1111/j.1462-2920.2010.02294.x

Bortolaia, V., Mander, M., Jensen, L. B., Olsen, J. E., and Guardabassi, L. (2015). Persistence of vancomycin resistance in multiple clones of *Enterococcus faecium* isolated from Danish broilers 15 years after the ban of avoparcin. *Antimicrob. Agents Chemother.* 59, 2926–2929. doi: 10.1128/AAC.05072-14

Brandl, K., Plitas, G., Mihu, C. N., Ubeda, C., Jia, T., Fleisher, M., et al. (2008). Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature* 455, 804–807. doi: 10.1038/nature07250

Brown, A. T., and Wittenberger, C. L. (1971). Mechanism for regulating the distribution of glucose carbon between the Embden-Mayerhof and hexose-monophosphate pathways in *Streptococcus* faecalis. *J. Bacteriol.* 106, 456–467. doi: 10.1128/JB.106.2.456-467.1971

Bryant, M. P., and Small, N. (1956). The anaerobic monotrichous butyric acid-producing curved ros-shaped bacteria of the rumen. *J. Bacteriol.* 72, 16–21. doi: 10.1128/JB.72.1.16-21.1956

Cabral, M. E., Mukdsi, M. A. C., Medina de Figueroa, R. X., and Gonzalez, S. N. (2007). Citrate metabolism by *Enterococcus faecium* and *Enterococcus durans* isolated from goat's and ewe's milk: influence of glucose and lactose. *Can. J. Microbiol.* 53, 607–615. doi: 10.1139/W07-011

Calcaterra, C., Sfondrini, L., Rossini, A., Sommariva, M., Rumio, C., Ménard, S., et al. (2008). Critical role of TLR9 in acute graft-versus-host disease. *J. Immunol.* 181, 6132–6139. doi: 10.4049/jimmunol.181.9.6132

Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., et al. (2007). Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56, 1761–1772. doi: 10.2337/db06-1491

Chakrabarti, S., Lees, A., Jones, S. G., and Milligan, D. W. (2000). *Clostridium difficile* infection in allogeneic stem cell transplant recipients is associated with severe graft-versus-host disease and nonrelapse mortality. *Bone Marrow Transplant.* 26, 871–876. doi: 10.1038/sj.bmt.1702627

Clark, B., and McKendrick, M. (2004). A review of viral gastroenteritis. *Curr. Opin. Infect. Dis.* 17, 461–469. doi: 10.1097/00001432-200410000-00011

Clausen, M. R., and Mortensen, P. B. (1994). Kinetic studies on the metabolism of short-chain fatty acids and glucose by isolated rat colonocytes. *Gastroenterology* 106, 423–432. doi: 10.1016/0016-5085(94)90601-7

Collado, M. C., Rautava, S., Aakko, J., Isolauri, E., and Salminen, S. (2016). Human gut colonisation may be initiated *in utero* by distinct microbial communities in the placenta and amniotic fluid. *Sci. Rep.* 6:23129. doi: 10.1038/srep23129

Cummings, J. H., and Macfarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. *J. Appl. Bacteriol.* 70, 443–459. doi: 10.1111/j.1365-2672.1991.tb02739.x

Cummings, J. H., Pomare, E. W., Branch, W. J., Naylor, C. P. E., and MacFarlane, G. T. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 28, 1221–1227. doi: 10.1136/gut.28.10.1221

De Vuyst, L., and Leroy, F. (2011). Cross-feeding between Bifidobacteria and butyrate-producing colon bacteria explains bifdobacterial competitiveness, butyrate production, and gas production. *Int. J. Food Microbiol.* 149, 73–80. doi: 10.1016/j.ijfoodmicro.2011.03.003

Deleemans, J. M., Chleilat, F., Reimer, R. A., Henning, J. W., Baydoun, M., Piedalue, C. A., et al. (2019). The chemo-gut study: investigating the long-term effects of chemotherapy on gut microbiota, metabolic, immune, psychological and cognitive parameters in young adult cancer survivors; study protocol. *BMC Cancer* 19:1243. doi: 10.1186/s12885-019-6473-8

Delia, P., Sansotta, G., Donato, V., Messina, G., Frosina, P., Pergolizzi, S., et al. (2002). Prevention of radiation-induced diarrhea with the use of VSL#3, a new high-potency probiotic preparation. *Am. J. Gastroenterol.* 97, 2150–2152. doi: 10.1111/j.1572-0241.2002.05946.x

Della Ragione, F., Criniti, V., Della Pietra, V., Borriello, A., Oliva, A., Indaco, S., et al. (2001). Genes modulated by histone acetylation as new effectors of butyrate activity. *FEBS Lett.* 499, 199–204. doi: 10.1016/S0014-5793(01)02539-X

Devaux, C. A., Mezouar, S., and Mege, J. L. (2019). The E-cadherin cleavage associated to pathogenic bacteria infections can favor bacterial invasion and transmigration, dysregulation of the immune response and cancer induction in humans. *Front. Microbiol.* 10:2598. doi: 10.3389/fmicb.2019.02598

Devaux, C., and Raoult, D. (2018). The microbiological memory, an epigenetic regulator governing the balance between good health and metabolic disorders. *Front. Microbiol.* 9:1379. doi: 10.3389/fmicb.2018.01379

Dubberke, E. R., Sadhu, J., Gatti, R., Reske, K. A., DiPersio, J. F., Devine, S. M., et al. (2007). Severity of *Clostridium difficile*-associated disease (CDAD) in allogeneic stem cell transplant recipients: evaluation of a CDAD severity grading system. *Infect. Control Hosp. Epidemiol.* 28, 208–211. doi: 10.1086/511792

Dubin, K., Callahan, M. K., Ren, B., Khanin, R., Viale, A., Ling, L., et al. (2016). Intestinal microbiome analyses identify melanoma patients at risk for checkpoint-blockade-induced colitis. *Nat. Commun.* 7:10391. doi: 10.1038/ncomms10391

Duncan, S. H., Barcenilla, A., Stewart, C. S., Pryde, S. E., and Flint, H. J. (2002). Acetate utilization and butyryl coenzyme A (CoA): acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. *Appl. Environ. Microbiol.* 68, 5186–5190. doi: 10.1128/AEM.68.10.5186-5190.2002

Duncan, S. H., Louis, P., and Flint, H. J. (2004). Lactate-utilizing bacteria, isolated from human faeces, that produce butyrate as a major fermentation product. *Appl. Environ. Microbiol.* 70, 5810–5817. doi: 10.1128/AEM.70.10.5810-5817.2004

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005). Diversity of the human intestinal microbial flora. *Science* 308, 1635–1638. doi: 10.1126/science.1110591

Elkrief, A., Derosa, L., Zitvogel, L., Kroemer, G., and Routy, B. (2019). The intimate relationship between gut microbiota and cancer immunotherapy. *Gut Microbes* 10, 424–428. doi: 10.1080/19490976.2018

Elshaghabee, F. M. F., Bockelmann, W., Meske, D., Vrese, M. D., Walte, H. G., Schrezenmeir, J., et al. (2016). Ethanol production by selected intestinal microorganisms and lactic acid bacteria growing under different nutritional conditions. *Front. Microbiol.* 7:47. doi: 10.3389/fmicb.2016.00047

Falony, G., Calmeyn, T., Leroy, F., and De Vuyst, L. (2009). Coculture fermentations of *Bifidobacterium* species and *Bacteroides* thetaiotaomicron reveal a mechanistic insight into the prebiotic effect of inulin-type fructans. *Appl. Environ. Microbiol.* 75, 2312–2319. doi: 10.1128/AEM.02649-08

Falony, G., Vlachou, A., Verbrugghe, K., and De Vuyst, L. (2006). Cross-feeding between *Bifidobacterium longum* BB536 and acetate-converting, butyrate-producing colon bacteria during growth on oligofructose. *Appl. Environ. Microbiol.* 72, 7835–7841. doi: 10.1128/AEM.01296-06

Feghoul, L., Chevret, S., Cuinet, A., Dalle, J. H., Ouachée, M., Yacouben, K., et al. (2015). Adenovirus infection and disease in paediatric haematopoietic stem cell transplant patients: clues for antiviral pre-emptive treatment. *Clin. Microbiol. Infect.* 21, 701–709. doi: 10.1016/j.cmi.2015.03.011

Ford, C. D., Gazdik, M. A., Lopansri, B. K., Webb, B., Mitchell, B., Coombs, J., et al. (2017). Vancomycin-resistant *Enterococcus* colonization and bacteremia and hematopoietic stem-cell transplantation outcomes. *Biol. Blood Marrow Transplant.* 23, 340–346. doi: 10.1016/j.bbmt.2016.11.017

Fujiwara, H., Docampo, M. D., Riwes, M., Peltier, D., Toubai, T., Henig, I., et al. (2018). Microbial metabolite sensor GPR43 controls severity of experimental GVHD. *Nat. Commun.* 9:3674. doi: 10.1038/s41467-018-06048-w

Gavaldà-Navarro, A., Hondares, E., Giralt, M., Mampel, T., Iglesias, R., and Villarroya, F. (2015). Fibroblast growth factor 21 in breast milk controls neonatal intestine function. *Sci. Rep.* 5:13717. doi: 10.1038/srep13717

Golob, J. L., DeMeules, M. M., Loeffelhoz, T., Quinn, Z. Z., Dame, M. K., Silvestri, S. S., et al. (2019). Butyrogenic bacteria after acute graft-versus-host disease (GVHD) are associated with the development of steroid-refractory GVHD. *Blood Adv.* 3, 2866–2869. doi: 10.1182/bloodadvances.2019000362

Gong, J., Chehrazi-Raffle, A., Placencio-Hickok, V., Guan, M., Hendifar, A., and Salgia, R. (2019). The gut microbiome and response to immune checkpoint inhibitors: preclinical and clinical strategies. *Clin. Transl. Med.* 8:9. doi: 10.1186/s40169-019-0225-x

Gopalakrishnan, V., Spencer, C. N., Nezi, L., Reuben, A., Andrews, M. C., Karpinets, T. V., et al. (2018). Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* 359, 97–103. doi: 10.1126/scien ce.aan4236

Grill, J. P., Crociani, J., and Ballongue, J. (1995). Characterization of fructose 6 phosphate phosphoketolases purified from *Bifidobacterium* species. *Curr. Microbiol.* 31, 49–54. doi: 10.1007/BF00294634

Han, J., Wang, Y., Song, D., Lu, Z., Dong, Z., Miao, H., et al. (2018). Effects of *Clostridium butyricum* and *Lactobacillus plantarum* on growth performance, immune function and volatile fatty acid level of caecal digesta in broilers. *Food Agric. Immunol.* 29, 797–807. doi: 10.1080/09540105.2018.1457013

Heimesaat, M. M., Nogai, A., Bereswill, S., Plickert, R., Fischer, A., Loddenkemper, C., et al. (2010). MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. *Gut* 59, 1079–1087. doi: 10.1136/gut.2009.197434

Heinken, A., Khan, M. T., Paglia, G., Rodionov, D. A., Harmsen, H. J. M., and Thiele, I. (2014). Functional metabolic map of *Faecalibacterium prausnitzii*, a beneficial human gut microbe. *J. Bacteriol.* 196, 3289–3302. doi: 10.1128/JB.01780-14

Higgins, J. M., Cemadas, M., Tan, K., Irie, A., Wang, J., Takada, Y., et al. (2000). The role of alpha and beta chains in ligand recognition by beta 7 integrins. *J. Biol. Chem.* 275, 25652–25664. doi: 10.1074/jbc.M001228200

Hijova, E., and Chmelarova, A. (2007). Short chain fatty acids and colonic health. *Bratisl. Lek. Listy* 108, 354–358.

Hill, G. R., Teishima, T., Rebel, V. I., Krijanovski, O. I., Cooke, K. R., Brinson, Y. S., et al. (2000). The p55 TNF-α receptor plays a critical role in T cell alloreactivity. *J. Immunol.* 164, 656–663. doi: 10.4049/jimmunol.164.2.656

Hocquart, M., Lagier, J. C., Cassir, N., Saidani, N., Eldin, C., Kerbai, J., et al. (2018). Early fecal microbiota transplantation improves survival in severe *Clostridium difficile* infections. *Clin. Infect. Dis.* 66, 645–650. doi: 10.1093/cid/cix762

Holler, E., Butzhammer, P., Schmid, K., Hundsrucker, C., Koestler, J., Peter, K., et al. (2014). Metagenomic analysis of the stool microbiome in patients receiving allogeneic stem cell transplantation: loss of diversity is associated with use of systemic antibiotics and more pronounced in gastrointestinal graft-versus-host disease. *Biol. Blood Marrow Transplant.* 20, 640–645. doi: 10.1016/j.bbmt.2014.01.030

Hooper, L. V., and Macpherson, A. J. (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat. Rev. Immunol.* 10, 159–169. doi: 10.1038/nri2710

Hossain, M. S., Jaye, D. L., Pollack, B. P., Farris, A. B., Tselanyane, M. L., David, E., et al. (2011). Flagellin, a TLR5 agonist, reduces graft-versus-host disease in allogeneic hematopoietic stem cell transplantation recipients while enhancing antiviral immunity. *J. Immunol.* 187, 5130–5140. doi: 10.4049/jimmunol.1101334

Huang, T., Ding, M., Bergholdt, H. K. M., Wang, T., Heianza, Y., Sun, D., et al. (2018). Dairy consumption and body mass index among adults: mendelian randomization analysis of 184802 individuals from 25 studies. *Clin. Chem.* 64, 183–191. doi: 10.1373/clinchem.2017.280701

Huang, Y., Yang, W., Liu, H., Duan, J., Zhang, Y., Liu, M., et al. (2012). Effect of high-dose methotrexate chemotherapy on intestinal Bifidobacteria, *Lactobacillus* and *Escherichia coli* in children with acute lymphoblastic leukemia. *Exp. Biol. Med. (Maywood)* 237, 305–311. doi: 10.1258/ebm.2011.011297

Hullahalli, K., Rodrigues, M., Nguyen, U. T., and Palmer, K. (2018). An attenuated CRISP-Cas system in *Enterococcus faecalis* permits DNA acquisition. *mBio* 9, e00414–e00418. doi: 10.1128/mBio.00414-18

Human Microbiome Project Consortium (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234

Huycke, M. M., Abrams, V., and Moore, D. R. (2002). *Enterococcus faecalis* produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. *Carcinogenesis* 23, 529–536. doi: 10.1093/carcin/23.3.529

Imado, T., Iwasaki, T., Kitano, S., Satake, A., Kuroiwa, T., Tsunemi, S., et al. (2010). The protective role of host toll-like receptor-4 in acute graft-versus-host disease. *Transplantation* 90, 1063–1070. doi: 10.1097/TP.0b013e3181f86947

Jenq, R. R., Taur, Y., Devlin, S. M., Ponce, D. M., Goldberg, J. D., Ahr, K. F., et al. (2015). Intestinal *Blautia* is associated with reduced death from graft-versus-host disease. *Biol. Blood Marrow Transplant.* 21, 1373–1383. doi: 10.1016/j.bbmt.2015.04.016

Jones, B. V., Begley, M., Hill, C., Gahan, C. G., and Marchesi, J. R. (2008). Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. *Proc. Natl. Acad. Sci. U. S. A.* 105, 13580–13585. doi: 10.1073/pnas.0804437105

Jorsal, T., Rhee, N. A., Pedersen, J., Wahlgren, C. D., Mortensen, B., Jepsen, S. L., et al. (2018). Endocrine K and L cells in healthy and type 2 diabetic individuals. *Diabetologia* 61, 284–294. doi: 10.1007/s00125-017-4450-9

Juajun, O., Nguyen, T. H., Maischberger, T., Iqbal, S., Haltrich, D., and Yammabhai, M. (2011). Cloning, purification, and characterization of β-galactosidase from *Bacillus licheniformis* DSM 13. *Appl. Microbiol. Biotechnol.* 89, 645–654. doi: 10.1007/s00253-010-2862-2

Kaito, S., Toya, T., Yoshifugi, K., Kurosawa, S., Inamoto, K., Takeshita, K., et al. (2018). Fecal microbiota transplantation with frozen capsules for a patient with refractory acute gut graft-versus-host disease. *Blood Adv.* 2, 3097–3101. doi: 10.1182/bloodadvances.2018024968

Kastl, A. J. Jr., Terry, N., Wu, G. D., and Albenberg, L. G. (2020). The structure and function of the human small intestinal microbiota: current understanding and future directions. *Cell. Mol. Gastroenterol. Hepatol.* 9, 33–45. doi: 10.1016/j.jcmgh.2019.07.006

Kiczorowska, B., Samolinska, W., Mustafa Al-Yasiry, A. R., Kiczorowski, P., and Winiarska-Mieczan, A. (2017). The natural feed additives as immunostimulants in monogastric animal nutrition—a review. *Ann. Anim. Sci.* 17, 605–625. doi: 10.1515/aoas-2016-0076

Kim, M. S., Park, E. J., Roh, S. W., and Bae, J. W. (2011). Diversity and abundance of single-stranded DNA viruses in human feces. *Appl. Environ. Microbiol.* 77, 8062–8070. doi: 10.1128/AEM.06331-11

Köhler, N., and Zeiser, R. (2019). Intestinal microbiota influence immune tolerance post allogeneic hematopoietic cell transplantation and intestinal GVHD. *Front. Immunol.* 9:3179. doi: 10.3389/fimmu.2018.03179

Korbling, M., and Freireich, E. J. (2011). Twenty-five years of peripheral blood stem cell transplantation. *Blood* 117, 6411–6416. doi: 10.1182/blood-2010-12-322214

Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Bäckhed, H. K., et al. (2012). Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell* 150, 470–480. doi: 10.1016/j.cell.2012.07.008

Kowaloff, E. M., Phang, J. M., Granger, A. S., and Downing, S. J. (1977). Regulation of proline oxidase activity by lactate. *Proc. Natl. Acad. Sci. U. S. A.* 74, 5368–5371. doi: 10.1073/pnas.74.12.5368

Kumagai, T., Rahman, F., and Smith, A. M. (2018). The microbiome and radiation induced-bowelinjury: evidence for potential mechanistic role in disease pathogenesis. *Nutrients* 10:1405. doi: 10.3390/nu10101405

Kurosu, H., Choi, M., Ogawa, Y., Dickson, A. S., Goetz, R., Eliseenkova, A. V., et al. (2007). Tissue-specific expression of betaKlotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activity of FGF19 and FGF21. *J. Biol. Chem.* 282, 26687–26695. doi: 10.1074/jbc.M704165200

Kuwahara, A. (2014). Contributions of colonic short-chain fatty acid receptors in energy homeostasis. *Front. Endocrinol.* 5:144. doi: 10.3389/fendo.2014.00144

Lagier, J. C., Khelaifia, S., Alou, M. T., Ndongo, S., Dione, N., Hugon, P., et al. (2016). Culture of previously uncultured members of the human gut microbiota by culturomics. *Nat. Microbiol.* 1:16203. doi: 10.1038/nmicrobiol.2016.203

Lecerf, J. M., Dépeint, F., Clerc, E., Dugenet, Y., Niamba, C. N., Rhazi, L., et al. (2012). Xylo-oligosaccharide (XOS) in combination with inulin modulates both the intestinal environment and immune status in healthy subjects, while XOS alone only shows prebiotic properties. *Br. J. Nutr.* 108, 1847–1858. doi: 10.1017/S0007114511007252

Legoff, J., Resche-Rigon, M., Bouquet, J., Robin, M., Naccache, S. N., Mercier-Delarue, S., et al. (2017). The eukaryotic gut virome in hematopoietic stem cell transplantation: new clues in enteric graft-versus-host disease. *Nat. Med.* 23, 1080–1085. doi: 10.1038/nm.4380

Liao, S. F., and Nyachoti, C. M. (2017). Using probiotics to improve swine gut health and nutrient utilization. *Anim. Nutr.* 3, 331–343. doi: 10.1016/j.aninu.2017.06.007

Littman, D. R., and Pamer, E. G. (2011). Role of the commensal microbiota in normal and pathogenic host immune responses. *Cell Host Microbe* 10, 311–323. doi: 10.1016/j.chom.2011.10.004

Liu, L., Zhang, L., Tang, W., Gu, Y., Hua, Q., Yang, S., et al. (2012). Phosphoketolase pathway for xylose catabolism in *Clostridium acetobutylicum* revealed by 13C metabolic flux analysis. *J. Bacteriol.* 194, 5413–5422. doi: 10.1128/JB.00713-12

Llorente, C., Jepsen, P., Inamine, T., Wang, L., Bluemel, S., Wang, H. J., et al. (2017). Gastric acid suppression promotes alcoholic liver disease by inducing overgrowth of intestinal *Enterococcus*. *Nat. Commun.* 8:837. doi: 10.1038/s41467-017-00796-x

Long, C., Liu, Y., He, L., Tan, Q., Yu, Z., Xiao, N., et al. (2018). Bacterial lactase genes diversity in intestinal mucosa of mice with dysbacterial diarrhea induced by antibiotics. *Biotech* 8:176. doi: 10.1007/s13205-018-1191-5

Luo, W. X., Xie, M., and Gao, L. W. (2016). Observation on the therapeutic effect of lactase on lactose intolerance in infants with diarrhea. *Strait Pharm. J.* 28, 153–154.

Luu, M., Weigand, K., Wedi, F., Breidenbend, C., Leister, H., Pautz, S., et al. (2018). Regulation of the effector function of CD8+ T cells by gut microbiota-derived metabolite butyrate. *Sci. Rep.* 8:14430. doi: 10.1038/s41598-018-32860-x

Magenau, J., Runaas, L., and Reddy, P. (2016). Advances in understanding the pathogenesis of graft-versus-host disease. *Br. J. Haematol.* 173, 190–205. doi: 10.1111/bjh.13959

Maharshak, N., Hub, E. Y., Paiboonrungruang, C., Shanahan, M., Thurlow, L., Herzog, J., et al. (2015). *Enterococcus faecalis* gelatinase mediates intestinal permeability via protease-activated receptor 2. *Infect. Immun.* 83, 2762–2770. doi: 10.1128/IAI.00425-15

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., et al. (2006). Comparative genomics of the lactic acid bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 103, 15611–15616. doi: 10.1073/pnas.0607117103

Manfredo Vieira, S., Hiltensperger, M., Kumar, V., Zegarra-Ruiz, D., Dehner, C., Khan, N., et al. (2018). Translocation of a gut pathobiont drives autoimmunity in mice and humans. *Science* 360, 1156–1161. doi: 10.1126/science.aar7201

Markwick, L. J. L., Riva, A., Ryan, J. M., Cooksley, H., Palma, E., Tranah, T. H., et al. (2015). Blockade of PD1 and TIM3 restores innate and adaptive immunity in patients with acute alcoholic hepatitis. *Gastroenterology* 148, 590–602. doi: 10.1053/j.gastro.2014.11.041

Mathewson, N. D., Jenq, R., Mathew, A. V., Koenigsknecht, M., Hanash, A., Toubai, T., et al. (2016). Gut microbiome derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. *Nat. Immunol.* 17, 505–513. doi: 10.1038/ni.3400

Meisel, M., Hinterleitner, R., Pacis, A., Chen, L., Earley, Z. M., Mayassi, T., et al. (2018). Microbial signals drive pre-leukaemic myeloproliferation in a *Tet2*-deficeint host. *Nature* 557, 580–584. doi: 10.1038/s41586-018-0125-z

Merli, M., Lucidi, C., Di Gregorio, V., Giannelli, V., Giusto, M., Ceccarelli, G., et al. (2015). The chronic use of beta-blockers and proton pump inhibitors may affect the rate of bacterial infections in cirrhosis. *Liver Int.* 35, 362–369. doi: 10.1111/liv.12593

Miller, T. L., and Wolin, M. J. (1979). Fermentations by saccharolytic intestinal bacteria. *Am. J. Clin. Nutr.* 32, 164–172. doi: 10.1093/ajcn/32.1.164

Million, M., Armstrong, N., Khelaifia, S., Guilhot, E., Richez, M., Lagier, J. C., et al. (2020). The antioxidants glutathione, ascorbic acid and uric acid maintain butyrate production by human gut *Clostridia* in the presence of oxygen. *Sci. Rep.* 10:7705. doi: 10.1038/s41598-020-64834-3

Million, M., Tidjani Alou, M., Khelaifia, S., Bachar, D., Lagier, J. C., Dione, N., et al. (2016). Increased gut redox and depletion of anaerobic and methanogenic prokaryotes in severe acute malnutrition. *Sci. Rep.* 6:26051. doi: 10.1038/srep26051

Million, M., Tomas, J., Wagner, C., Lelouard, H., Raoult, D., and Gorvel, J. P. (2018). New insights in gut microbiota and mucosal immunity of the small intestine. *Hum. Microb. J.* 7-8, 23–32. doi: 10.1016/j.humic.2018.01.004

Minot, S., Sinha, R., Chen, J., Li, H., Keilbaugh, S. A., Wu, G. D., et al. (2011). The human gut virome: inter-individual variation and dynamic response to diet. *Genome Res.* 21, 1616–1625. doi: 10.1101/gr.122705.111

Misselwitz, B., Butter, M., Verbeke, K., and Fox, M. R. (2019). Update on lactose malabsorption and intolerance: pathogenesis, diagnosis and clinical management. *Gut* 68, 2080–2091. doi: 10.1136/gutjnl-2019-318404

Murai, M., Yoneyama, H., Ezaki, T., Suematsu, M., Terashima, Y., Harada, A., et al. (2003). Peyer's patch is the essential site in initiating murine acute and lethal graft-versus-host reaction. *Nat. Immunol.* 4, 154–160. doi: 10.1038/ni879

Musa, A. E., Shabeeb, D., and Alhilfi, H. S. Q. (2019). Protective effect of melatonin against radiotherapy-induced small intestinal oxidative stress: biochemical evaluation. *Medicina* 55:308. doi: 10.3390/medicina55060308

Nalle, S. C., Zuo, L., Ong, M. L. D. M., Singh, G., Worthylake, A. M., Choi, W., et al. (2019). Graft-versus-host disease propagation increased intestinal epithelial thight junction permeability. *J. Clin. Invest.* 129, 902–914. doi: 10.1172/JCI98554

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., et al. (2012). Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267. doi: 10.1126/science.1223813

Nohr, M. K., Pedersen, M. H., Gille, A., Egerod, K. L., Engelstoft, M. S., Husted, A. S., et al. (2013). GPR41/FFAR3 and GPR43/FFAR2 as cosensors for short-chain fatty acids in enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes. *Endocrinology* 154, 3552–3564. doi: 10.1210/en.2013-1142

Noris, R. D., Mahamed, A. Z., Martin, J. M., and Docimo, S. G. (2010). *Enterococcus faecalis* cellulitis/fasciitis after hypospadias surgery. *Urology* 76, 107–108. doi: 10.1016/j.urology.2009.11.053

Paganelli, F. L., Huebner, J., Singh, K. V., Zhang, X., van Schaik, W., Wobser, D., et al. (2016). Genome-wide screening identifies phosphotransferases system permease BepA to be involved in *Enterococcus faecium* endocarditis and biofilm formation. *J. Infect. Dis.* 214, 189–195. doi: 10.1093/infdis/jiw108

Pande, S., Shitut, S., Freund, L., Westermann, M., Bertels, F., Colesie, C., et al. (2015). Metabolic cross-feeding via intercellular nanotubes among bacteria. *Nat. Commun.* 6:6238. doi: 10.1038/ncomms7238

Park, J., Goergen, C. J., HogenEsch, H., and Kim, C. H. (2020). Chronically elevated levels of short-chain fatty acids induce T cell-mediated ureteritis and hydronephrosis. *J. Immunol.* 196, 2388–2400. doi: 10.4049/jimmunol.1502046

Penack, O., Holler, E., and van den Brink, M. R. (2010). Graft-versus-host disease: regulation by microbe-associated molecules and innate immune receptors. *Blood* 115, 1865–1872. doi: 10.1182/blood-2009-09-242784

Pichereau, C., Desseaux, K., Janin, A., Scieux, C., Peffault de Latour, R., Whaard, A., et al. (2012). The complex relationship between human herpesvirus 6 and acute graft-versus-host disease. *Biol. Blood Marrow Transplant.* 18, 141–144. doi: 10.1016/j.bbmt.2011.07.018

Priya, R., Gomez, G. A., Budnar, S., Verma, S., Cox, H. L., Hamilton, N. A., et al. (2015). Feedback regulation through myosin II confers robustness on RhoA signalling at E-cadherin junctions. *Nat. Cell Biol.* 17, 1282–1293. doi: 10.1038/ncb3239

Priyadarshini, M., Kotlo, K. U., Dudeja, P. K., and Layden, B. T. (2019). Role of short chain fatty acid receptors in intestinal physiology and pathology. *Compr. Physiol.* 8, 1091–1115. doi: 10.1002/cphy.c170050

Qiang, Y., Xu, J., Yan, C., Jin, H., Xiao, T., Yan, N., et al. (2017). Butyrate and retinoic acid imprint mucosal-like dendritic cell development synergistically from bone marrow cells. *Clin. Exp. Immunol.* 189, 290–297. doi: 10.1111/cei.12990

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., et al. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464, 59–65. doi: 10.1038/nature08821

Qin, P., Zou, Y., Dai, Y., Luo, G., Zhang, X., and Xiao, L. (2019). Characterization a novel butyric acid-producing bacterium *Collinsella aerofaciens* subsp. *Shenzhenensis* subsp. Nov. *Microorganisms* 7:78. doi: 10.3390/microorganisms7030078

Rautava, S., Luoto, R., Salminen, S., and Isolauri, E. (2012). Microbial contact during pregnancy, intestinal colonization and human disease. *Nat. Rev. Gastroenterol. Hepatol.* 9, 565–576. doi: 10.1038/nrgastro.2012.144

Reissier, S., Saleh-Mghir, A., Guerin, F., Massias, L., Ghout, I., Sinel, C., et al. (2018). In vivo daptomycin efficacy against experimental vancomycin-resistant *Enterococcus faecium* endocarditis. *J. Antimicrob. Chemother.* 73, 981–986. doi: 10.1093/jac/dkx499

Rhimi, M., Aghajari, N., Jaouadi, B., Juy, M., Boudebbouze, S., Maquin, E., et al. (2009). Exploring the acidotolerance of betagalactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*: an attractive enzyme for lactose bioconversion. *Res. Microbiol.* 160, 775–784. doi: 10.1016/j.resmic.2009.09.004

Rios-Covian, D., Gueimonde, M., Duncan, S. H., Flint, H. J., and de los Reyes-Gavilan, C. G. (2015). Enhanced butyrate formation by cross-feeding between *Faecalibacterium prausnitzii* and *Bifidobacterium adolescentis*. *FEMS Microbiol. Lett.* 362:fnv176. doi: 10.1093/femsle/fnv176

Rivière, A., Selak, M., Lantin, D., Leroy, F., and De Vuyst, L. (2016). Bifidobacteria and butyrate-producing colon bacteria: importance and strategies for their stimulation in the human gut. *Front. Microbiol.* 7:979. doi: 10.3389/fmicb.2016.00979

Riwes, M., and Reddy, P. (2018). Microbial metabolites and graft versus host disease. *Am. J. Transplant.* 18, 23–29. doi: 10.1111/ajt.14443

Roberfroid, M. B. (2007). Inulin-type fructans: functional food ingredient. *J. Nutr.* 137, 2493S–2502S. doi: 10.1093/jn/137.11.2493S

Russo, P., Pia, M., Fiocco, D., Capozzi, V., Drider, D., and Spano, G. (2017). *Lactobacillus* plantarum with broad antifungal activity: a promising approach to increase safety and shelf-life of cereal-based products. *Int. J. Food Microbiol.* 247, 48–54. doi: 10.1016/j.ijfoodmicro.2016.04.027

Saha, S. K., Lee, S. B., Won, J., Choi, H. Y., Kim, K., Yang, G. M., et al. (2017). Correlation between oxidative stress, nutrition, and cancer initiation. *Int. J. Mol. Sci.* 18:1544. doi: 10.3390/ijms18071544

Sahin, U., Toprak, S. K., Atilla, P. A., Atilla, E., and Demirer, T. (2016). An overview of infectious complications after allogenic hematopoeitic stem cell transplantation. *J. Infect. Chemother.* 22, 505–514. doi: 10.1016/j.jiac.2016.05.006

Saito, H. E., Harp, J. R., and Fozo, E. M. (2018). *Enterococcus faecalis* responds to individual exogenous fatty acids independently of their degree of saturation or chain length. *Appl. Environ. Microbiol.* 84, e01633–e01717. doi: 10.1128/AEM.01633-17

Schwab, L., Goroncy, L., Palaniyandi, S., Gautam, S., Triantafyllopoulou, A., Mocsai, A., et al. (2014). Neutrophil granulocytes recruited upon translocation of intestinal bacteria enhance graft-versus-host disease via tissue damage. *Nat. Med.* 20, 648–654. doi: 10.1038/nm.3517

Schwartz, S., Vergoulidou, M., Schreier, E., Loddenkemper, C., Reinwald, M., Schmidt-Hieber, M., et al. (2011). Norovirus gastroenteritis causes severe and lethal complications after chemotherapy and hematopoietic stem cell transplantation. *Blood* 117, 5850–5856. doi: 10.1182/blood-2010-12-325886

Severyn, C. J., Brewster, R., and Andermann, T. M. (2019). Microbiota modification in hematology: still at the bench or ready for the bedside? *Blood Adv.* 3, 303–314. doi: 10.1182/bloodadvances.2019000365

Shanahan, F., van Sinderen, D., O'Toole, P. W., and Stanton, C. (2017). Feeding the microbiota: transducer of nutrient signals for the host. *Gut* 66, 1709–1717. doi: 10.1136/gutjnl-2017-313872

Shouval, R., Geva, M., Nagler, A., and Youngster, I. (2019). Fecal microbiota transplantation for treatment of acute graft-versus-host disease. *Clin. Hematol. Int.* 1, 28–35. doi: 10.2991/chi.d.190316.002

Si, X., Shang, W., Zhou, Z., Strappe, P., Wang, B., Bird, A., et al. (2018). Gut microbiome-induced shift of actetate to butyrate positively manages dysbiosis in high fat diet. *Mol. Nutr. Food Res.* 62:1700670. doi: 10.1002/mnfr.201700670

Steck, N., Hoffmann, M., Sava, I. G., Kim, S. C., Hahne, H., Tonkonogy, S. L., et al. (2011). *Enterococcus faecalis* metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. *Gastroenterology* 141, 959–971. doi: 10.1053/j.gastro.2011.05.035

Stein-Thoeringer, C. K., Nichols, K. B., Lazrak, A., Docampo, M. D., Slingerland, A. E., Slingerland, J. B., et al. (2019). Lactose drives *Enterococcus* expansion to promote graft-versus-host disease. *Science* 366, 1143–1149. doi: 10.1126/science.aax3760

Tamburini, F. B., Andermann, T. M., Tkachenko, E., Senchyna, F., Banaei, N., and Bhatt, A. S. (2018). Precision identification of diverse bloodstream pathogens in the gut microbiome. *Nat. Med.* 24, 1809–1814. doi: 10.1038/s41591-018-0202-8

Taur, Y., Jenq, R. R., Perales, M. A., Littmann, E. R., Morjaria, S., Ling, L., et al. (2014). The effects of intestinal tract bacterial diversity on mortality following allogenic hematopoietic stem cell transplantation. *Blood* 124, 1174–1182. doi: 10.1182/blood-2014-02-554725

Taur, Y., Xavier, J. B., Lipuma, L., Ubeda, C., Goldberg, J., Gobourne, A., et al. (2012). Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. *Clin. Infect. Dis.* 55, 905–914. doi: 10.1093/cid/cis580

Tezze, C., Romanello, V., and Sandri, M. (2019). FGF21 as modulator of metabolism in health and disease. *Front. Physiol.* 10:419. doi: 10.3389/fphys.2019.00419

Thalambedu, N., Khan, Y., Zhang, Q., Khanal, S., and Ashfaq, A. (2019). Immune-mediated colitis from dual checkpoint inhibitors. *Cureus* 11:e6233. doi: 10.7759/cureus.6233

Tidjani Alou, M., Million, M., Traore, S. I., Mouelhi, D., Khelaifia, S., Bachar, D., et al. (2017). Gut bacteria missing in severe acute malnutrition, can we identify potential probiotics by culturomics? *Front. Microbiol.* 8:899. doi: 10.3389/fmicb.2017.00899

Tien, B. Y. Q., Goh, H. M. S., Chong, K. K. L., Bhaduri-Tagore, S., Holec, S., Dress, R., et al. (2017). *Enterococcus faecalis* promotes innate immune suppression and polymicrobial catheter-associated urinary tract infection. *Infect. Immun.* 85. pii: e00378–e00417. doi: 10.1128/IAI.00378-17

Topping, D. L., and Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol. Rev.* 81, 1031–1064. doi: 10.1152/physrev.2001.81.3.1031

Tran, T. H. T., Everaert, N., and Bindelle, J. (2016). Review on the effects of potential prebiotics on controlling intestinal enteropathogens *Salmonella* and *Escherichia coli* in pig production. *J. Anim. Physiol. Anim. Nutr.* 102, 17–32. doi: 10.1111/jpn.12666

Tremorali, V., and Backhed, F. (2012). Functional interaction between the gut microbiota and host metabolism. *Nature* 489, 242–249. doi: 10.1038/nature11552

Trifilio, S. M., Pi, J., and Mehta, J. (2012). Changing epidemiology of *Clostridium difficile*-associated disease during stem cell transplantation. *Biol. Blood Marrow Transplant.* 19, 405–409. doi: 10.1016/j.bbmt.2012.10.030

Troelsen, J. T., Mitchelmore, C., Spodsberg, N., Jensen, A. M., Noren, O., and Sjöström, H. (1997). Regulation of lactase-phlorizin-hydrolase gene expression by the caudal-related homoeodomain protein Cdx-2. *Biochem. J.* 322, 833–838. doi: 10.1042/bj3220833

Tuohy, K. M., Kolida, S., Lustenberger, A. M., and Gibson, G. R. (2001). The prebiotic effects of biscuits containing partially hydrolysed guar gum and fructo-oligosaccharides—a human volunteer study. *Br. J. Nutr.* 86, 341–348. doi: 10.1079/BJN2001394

Turnbaugh, P. J., Quince, C., Faith, J. J., McHardy, A. C., Yatsunenko, T., Niazi, F., et al. (2010). Organismal, genetic, and transcriptional variation in the deeply sequenced gut microbiomes of identical twins. *Proc. Natl. Acad. Sci. U. S. A.* 107, 7503–7508. doi: 10.1073/pnas.1002355107

van Bekkum, D. W., Roodenburg, J., Heidt, P. J., and van der Waaij, D. (1974). Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora. *J. Natl. Cancer Inst.* 52, 401–404. doi: 10.1093/jnci/52.2.401

Varga, T., Czimmerer, Z., and Nagy, L. (2011). PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. *Biochim. Biophys. Acta* 1812, 1007–1022. doi: 10.1016/j.bbadis.2011.02.014

Vieco-Saiz, N., Belguesmia, Y., Raspoet, R., Auclair, E., Gancel, F., Kempf, I., et al. (2019). Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. *Front. Microbiol.* 10:57. doi: 10.3389/fmicb.2019.00057

Villa, N. Y., and McFadden, G. (2018). Virotherapy as potential adjunct therapy for graft-vs-host disease. *Curr. Pathobiol. Rep.* 6, 247–263. doi: 10.1007/s40139-018-0186-6

Vital, M., Chuang Howe, A., and Tiedje, J. M. (2014). Revealing the bacterial butyrate synthesis pathways by analyzing (meta) genomic data. *mBio* 5:e00889. doi: 10.1128/mBio.00889-14

Vreugdenhil, A. C., Rousseau, C. H., Hartung, T., Greve, J. W., van'tVeer, C., and Buurman, W. A. (2003). Lipopolysaccharide (LPS)-binding protein mediates LPS detoxification by chylomicrons. *J. Immunol.* 170, 1399–1405. doi: 10.4049/jimmunol.170.3.1399

Wagnerberger, S., Spruss, A., Kanuri, G., Volynets, V., Stahl, C., Bischoff, S. C., et al. (2012). Toll-like receptor 1-9 are elevated in livers with fructose-induced hepatic steatosis. *Br. J. Nutr.* 107, 1727–1738. doi: 10.1017/S0007114511004983

Waldecker, M., Kautenburger, T., Daumann, H., Busch, C., and Schrenk, D. (2008). Inhibition of histone-deacetylase activity by short-chain fatty acids and some polyphenol metabolites formed in the colon. *J. Nutr. Biochem.* 19, 587–593. doi: 10.1016/j.jnutbio.2007.08.002

Walter, J., and Ley, R. (2011). The human gut microbiome: ecology and recent evolutionary changes. *Annu. Rev. Microbiol.* 65, 411–429. doi: 10.1146/annurev-micro-090110-102830

Wang, Z., Wang, Q., Wang, X., Zhu, L., Chen, J., Zhang, B., et al. (2019). Gut microbial dysbiosis is associated with development and progression of radiation enteritis during pelvic radiotherapy. *J. Cell. Mol. Med.* 23, 3747–3756. doi: 10.1111/jcmm.14289

Weber, D., Jenq, R. R., Peled, J. U., Taur, Y., Hiergeist, A., Koestler, J., et al. (2017). Microbiota disruption induced by early use of broad-spectrum antibiotics is an independent risk factor of outcome after allogeneic stem cell transplantation. *Biol. Blood Marrow Transplant.* 23, 845–852. doi: 10.1016/j.bbmt.2017.02.006

Weber, D., Oefner, P. J., Hiergeist, A., Koestler, J., Gessner, A., Weber, M., et al. (2015). Low urinary indoxyl sulfate levels early after transplantation reflect a disrupted microbiome and are associated with poor outcome. *Blood* 126, 1723–1728. doi: 10.1182/blood-2015-04-638858

Wei, D., Heus, P., van de Wetering, F. T., van Tienhoven, G., Verleye, L., and Scholten, R. J. P. M. (2018). Probiotics for the prevention or treatment of chemotherapy or radiotherapy-related diarrhoea in people with cancer. *Cochrane Database Syst. Rev.* 8. Art.No: CD008831. doi: 10.1002/14651858.CD008831.pub3.

World Bank (2017). Drug-resistant infections: a threat to our economic future (Vol. 2): final report (English) number 114679, disclosure date April 08, 2017. HNP/Agriculture Global Antimicrobial Resistance Initiative. Washington, DC: World Bank Group. Available at: http://documents.worldbank.org/curated/en/323311493396993758/final-report

Yang, J. Y., Kim, M. S., Kim, E., Cheon, J. H., Lee, Y. S., Kim, Y., et al. (2016). Enteric viruses ameliorate gut inflammation via toll-like receptor 3 and toll-like receptor 7-mediated interferon-b production. *Immunity* 44, 889–900. doi: 10.1016/j.immuni.2016.03.009

Yue, X., Trifari, S., Aijö, T., Tsagaratou, A., Pastor, W. A., Zepeda-Martinez, J. A., et al. (2016). Control of Foxp3 stability through modulation of TET activity. *J. Exp. Med.* 213, 377–397. doi: 10.1084/jem.20151438

Zama, D., Bossu, G., Leardini, D., Muratore, E., Biagi, E., Prete, A., et al. (2020). Insights into the role of intestinal microbiota in hematopoietic stem-cell transplantation. *Ther. Adv. Hematol.* 11, 1–19. doi: 10.1177/2

Zerr, D. M., Boeckh, M., Delaney, C., Martin, P. J., Xie, H., Adler, A. L., et al. (2012). HHV-6 reactivation and associated sequelae after hematopoietic cell transplantation. *Biol. Blood Marrow Transplant.* 18, 1700–1708. doi: 10.1016/j.bbmt.2012.05.012

Zhang, T., Breitbart, M., Lee, W. H., Run, J. Q., Wei, C. L., Soh, S. W., et al. (2006). RNA viral community in human feces: prevalence of plant pathogenic viruses. *PLoS Biol.* 4:e3. doi: 10.1371/journal.pbio.0040003

Zhang, J., Song, L., Wang, Y., Liu, C., Zhang, L., Zhu, S., et al. (2019). Beneficial effect of butyrate-producing Lachnospiraceae on stress-induced visceral hypersensitivity in rats. *J. Gastroenterol. Hepatol.* 34, 1368–1376. doi: 10.1111/jgh.14536

Zheng, J., Wang, J., Pouliot, M., Authier, S., Loose, D. S., and Hauer-Jensen, M. (2015). Gene expression profiling in non-human primate jejunum, ileum and colon after total-body irradiation: a comparative study of segment-specific molecular and cellular responses. *BMC Genomics* 16:984. doi: 10.1186/s12864-015-2168-y

Zhu, Y. H., Li, X. Q., Zhang, W., Zhou, D., Liu, H. Y., and Wang, J. F. (2014). Dose-dependent effects of *Lactobacillus rhamnosus* on serum interleukin-17 production and intestinal T-cell responses in pig challenged with *Escherichia coli*. *Appl. Environ. Microbiol.* 80, 1787–1798. doi: 10.1128/AEM.03668-13

Zitvogel, L., and Kroemer, G. (2019). Immunostimulatory gut bacteria. *Science* 366, 1077–1078. doi: 10.1126/science.aaz7595

Zitvogel, L., Ma, Y., Raoult, D., Kroemer, G., and Gajewski, T. F. (2018). The microbiome in cancer immunotherapy: diagnostic tools and therapeutic stragies. *Science* 359, 1366–1370. doi: 10.1126/science.aar6918

Zoetendal, E. G., Raes, J., van den Bogert, B., Arumugam, M., Booijink, C. C., Troost, F. J., et al. (2012). The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. *ISME J.* 6, 1415–1426. doi: 10.1038/ismej.2011.212

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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Gingipain R1 and Lipopolysaccharide From *Porphyromonas gingivalis* Have Major Effects on Blood Clot Morphology and Mechanics

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Background: *Porphyromonas gingivalis* and its inflammagens are associated with a number of systemic diseases, such as cardiovascular disease and type 2 diabetes (T2DM). The proteases, gingipains, have also recently been identified in the brains of Alzheimer's disease patients and in the blood of Parkinson's disease patients. Bacterial inflammagens, including lipopolysaccharides (LPSs) and various proteases in circulation, may drive systemic inflammation.

Methods: Here, we investigate the effects of the bacterial products LPS from *Escherichia coli* and *Porphyromonas gingivalis*, and also the *P. gingivalis* gingipain [recombinant *P. gingivalis* gingipain R1 (RgpA)], on clot architecture and clot formation in whole blood and plasma from healthy individuals, as well as in purified fibrinogen models. Structural analysis of clots was performed using confocal microscopy, scanning electron microscopy, and AFM-Raman imaging. We use thromboelastography® (TEG®) and rheometry to compare the static and dynamic mechanical properties of clots.

Results: We found that these inflammagens may interact with fibrin(ogen) and this interaction causes anomalous blood clotting.

Conclusions: These techniques, in combination, provide insight into the effects of these bacterial products on cardiovascular health, and particularly clot structure and mechanics.

Keywords: Gingipain R1, lipopolysaccharide, *Porphyromonas gingivalis*, thromboelastography, rheometry, confocal, scanning electron microscopy, Raman

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BACKGROUND

Bacterial involvement in inflammatory conditions, via the occurrence of leaky gut (gut dysbiosis) and periodontitis and/or gingivitis, are accompanied by the shedding of cell wall components such as lipopolysaccharides (LPSs) and lipoteichoic acids (LTAs), and these molecules are known to be highly inflammagenic (1–7). The liberation of free iron is often an accompaniment

to inflammatory conditions, and we have brought these ideas and data together as the Iron Dysregulation and Dormant Microbes (IDDM) hypothesis of chronic inflammatory and cardiovascular diseases. Many non-communicable diseases have been associated with the presence of periodontitis, gut dysbiosis, bacterial translocation via the gut and increased levels of the bacterial inflammagen, lipopolysaccharide (LPS); for an overview see (5).

Diseases where bacterial involvement has been implicated include Alzheimer's disease (AD) and Parkinson's disease (PD), and disease development and progression has also been linked to periodontitis (8–16). Entrance of bacteria into the body might occur via gut dysbiosis, and impaired gut health is also present in both AD (15, 17) and PD (18–21). Type diabetes (T2DM) is also associated with bacterial translocation via the gut (22–24). LPS presence has also been noted in the central nervous system of patients with AD (25–27) and in their blood (28). LPS has also been implicated in T2DM (29, 30), sepsis (31), rheumatoid arthritis (32), and psoriasis vulgaris (33).

Porphyromonas gingivalis is a well-known bacterium that causes periodontitis and gingivitis (34, 35), and its inflammagens have been associated with the development of various inflammatory conditions (36–40). *P. gingivalis* and its inflammagens are associated with cardiovascular disease and T2DM (41). Except for the presence of its cell wall inflammagen LPS, *P. gingivalis* also produces an unique class of cysteine proteinases termed gingipains. Live *P. gingivalis,* as well as its LPS, are powerful peripheral and intracerebral inflammatory signaling initiators (42). LPS from *P. gingivalis* also acts via the Toll-like Receptor (TLR) signaling pathway the authors studied the TLR4 signaling pathway in C57BL/6 mice (43). Recently, Dominy and co-workers provided clear evidence that *P. gingivalis,* and more specifically, it protease, gingipains, play a fundamental role in the development of AD (40). They discovered gingipains in the brain lesions of AD patients (40). Gingipains consist of Arg-gingipain (Rgp) (RgpA and RgpB), and Lys-gingipain (Kgp), and play a central role in the virulence of this organism (44). Gingipains cleave proteins toward the C-terminal after arginine or lysine residues and are classified accordingly: gingipain R is arginine-specific and gingipain K is lysine-specific. This proteolytic activity of gingipains play a crucial role in the physiology of the bacterium, where it is essential for obtaining nutrients via protein degradation, for adherence to host surfaces and for further colonization (45). Gingipains are also known to exert fibrin(ogen)olytic activity and when present in circulation, can interact and cleave plasma proteins (46–48).

By definition, inflammation is normally accompanied by the production of inflammatory cytokines, such as interleukins (ILs) IL1β, IL6, and TNF-α, some bacterial inflammagens such as LPS are well-characterized, and more recently implicated in inflammatory conditions, but overall, little is known on how bacterial inflammagens act as biomarkers in the various inflammatory conditions (2, 4, 5). Inflammation is also an almost inevitable accompaniment of cardiovascular disease, but a much less recognized feature of inflammation is coagulopathies (49–51). We recently discovered that, in part, these coagulopathies were represented by the clotting of blood into an anomalous form, and that this can be catalyzed by miniscule amounts of LPS (from *E. coli*) or LTA (10^{-8} mol/mol fibrinogen) (2, 52). Recently, it was also noted that LPS from *P. gingivalis* added to platelets cause significant morphological changes to platelets (53). Platelets exposed to this LPS showed spreading, with increased presence of actin-rich filopodia, by activation of Cdc42, the small GTPase responsible for filopodia formation. Exposure of whole blood samples to LPS from *P. gingivalis* also significantly reduced clotting times (53).

Because of the findings of Dominy et al. where they detected gingipains in AD brain lesions (40) and our interest on how bacterial inflammagens interact with clotting proteins, we searched for the presence of gingipains in the serum of patients with PD (54). We detected RgpA from *P. gingivalis* in PD plasma using fluorescent antibodies and found significantly increased levels of this protease compared to age-matched controls.

Because there are numerous reports that *P.gingivalis* and its inflammagens are important contributary agents in neuroinflammatory, as well as cardiovascular conditions, including T2DM, the question now arose as to how gingipains and LPS from *P. gingivalis* interact with circulating plasma proteins. Microbial translocation from inflamed periodontal pockets into coronary atheroma via systemic circulation is also one of the proposed pathways that links periodontitis and myocardial infarction (55). We therefore seek to get specific answers with regards to their effects on both morphology and mechanics of clots. Therefore, in the present study, we investigate the effects of the bacterial products LPS from *E. coli* and *P. gingivalis*, as well as the gingipain RgpA *P. gingivalis* [recombinant *P. gingivalis* Gingipain R1 (RgpA)], on clot architecture and clot formation in whole blood and plasma from healthy individuals, as well as in purified fibrinogen models. Structural analysis of clots was performed using confocal microscopy, scanning electron microscopy and AFM-Raman imaging. We use thromboelastography® (TEG®) and rheometry to compare the static and dynamic mechanical properties of clots.

To investigate our hypothesis, the various analyses were done in various laboratories. We therefore included a large variety of equipment and sample preparation methods and used optimized and well-established protocols from each laboratory. These

Abbreviations: LPS, lipopolysaccharides; LTA, lipoteichoic acids; IDDM, Dysregulation and Dormant Microbes; AD, Alzheimer's disease; PD, Parkinson's disease; T2DM, Type diabetes; P. gingivalis, Porphyromonas gingivalis; TLR4, Toll-like Receptor 4; Rgp, Arg-gingipain; Kgp, Lys-gingipain; Rgp(A) and (B), arginine gingipains and recombinant *P. gingivalis* gingipain R1 (RgpA); ILs, Interleukins (ILs); E. coli, Escherichia coli; PPP, Platelet poor plasma; PDP, platelet-depleted plasma; HMDS, hexamethyldisilazane; OsO_4, osmium tetroxide; TEG®, Thrombelastograph®; LVE, linear viscoelastic behavior; J'_M, minimum-strain compliance; J'_L, large-strain compliance.

various techniques in combination provide insight into the effects of these bacterial products on coagulation, and particularly clot structure and mechanics. We found that these inflammagens may interact with fibrin(ogen) and cause blood to clot abnormally (anomalous clotting). These results are in line with our previous findings of LPS from *E. coli,* and we further show here that LPS from *E. coli* influences the clot structure of purified fibrin(ogen) (2). Furthermore, understanding how bacterial inflammagens interact with plasma proteins, when in circulation, may result in a better understanding of clot and coagulation pathologies in inflammatory conditions. Ultimately, we may find solutions to treat pathological clotting, driven by bacterial inflammagens, as pathological clotting is an important co-morbidity to most inflammatory conditions.

MATERIALS AND METHODS

Study Design and Ethical Statement

The present study uses a cross-sectional study design. Ethical clearance was obtained from the Health Research Ethics Committee (HREC) of Stellenbosch University, South Africa (N19/03/043) and from the Ethics Committee of the Medical University Vienna, Austria (EK1371/2015). Written informed consent was obtained from all participants followed by whole blood sampling. Study participants received a unique number that was used to guarantee anonymity throughout this study, and researchers followed Good Clinical Practice and guidelines from the ethics committee.

LPS and Gingipain (RgpA)

The bacterial analytes that were added to plasma and fibrinogen were prepared in endotoxin-free water and they are:

- RgpA (Abcam. ab225982); purity is at >90% SDS-PAGE
- *E. coli* LPS (Sigma, L2630) and *P. gingivalis* LPS (Sigma SMB00610). Both the LPSs' purity is MQ300, which is stipulated for products used in applications requiring enhanced change control and quality agreement. However, it is noted that Jain et al. (56) reported that some LPS preparations might have lipoprotein contaminants present.

Purified Fibrin(Ogen) Clot Model

We used three purified fibrin(ogen) clot models: (1) fluorescent fibrinogen conjugated to Alexa Fluor^{TM}488 (ThermoFisher, F13191), (2) non-conjugated purified fibrinogen (Sigma, F3879) and (3) non-conjugated purified fibrinogen depleted of von Willebrand factor, plasminogen, and fibronectin (CoaChrom, HFG3). These products were also prepared in endotoxin-free water.

Participants and Blood Collection

Healthy volunteers [N = 39; 23 females, 16 males; median age (interquartile range): 42] were recruited for this study. The inclusion criteria for healthy volunteers were: non-smokers, absence of infection, no use of anti-inflammatory or chronic medication, and no previous history of thrombotic disease, neurological diseases like AD and PD, or T2DM. An exclusion criterion was the presence of both gingivitis and periodontitis. Blood was drawn in serum-separating, EDTA, and sodium citrate tubes by a phlebotomist. After the blood was drawn, whole blood samples were allowed to rest for 30 min at room temperature before further processing for experimentation. Two plasma derivatives were created. Platelet poor plasma (PPP) was created by centrifuging whole blood at 3,000 g for 15 min. The plasma fraction was collected and stored at −80°C until experimentation. For rheometry analysis, platelet-depleted plasma (PDP) was created by centrifuging whole blood at 325 g for 8 min. After removal of the surface layer, the top two-thirds of this plasma were collected and centrifuged a second time at 2,310 g for 30 min, and the top two-thirds ultimately used for experimentation.

Scanning Electron Microscopy

Platelet Poor Plasma With LPS

A scanning electron microscope was used to view the ultrastructural changes of clots. PPP was exposed to LPS from *P. gingivalis* (n = 10; 10 ng.L^{-1}; 30 min) before creating a plasma clot on a 10 mm glass cover slip with the addition of thrombin (7 U·mL^{-1}). South African National Blood Service. Matching naïve clots were prepared with addition of thrombin. The samples were then washed in PBS followed by a fixation step of 4% formaldehyde and secondary fixation in 1% osmium tetroxide (OsO_4), with PBS wash steps in between. This was followed by serial dehydration in ethanol and hexamethyldisilazane (HMDS). The samples were coated with carbon and viewed with a Zeiss MERLIN FE-SEM with the InLens detector at 1 kV. All SEM images (3 images per clot) were analyzed using ImageJ where fibrin fiber width was assessed for each image using a grid overlay to accurately record these measurements. The central fibers in 12 squares on each image were measured.

Purified Fibrinogen With LPS and RgpA

Purified fibrinogen (CoaChrom) was incubated with the following substances and prepared in technical triplicate following the above protocol, with the exception of using 0.15 U·mL^{-1} alpha-thrombin (CoaChrom, HF2A) to create the clots. Samples were viewed as above.

- RgpA at 20 μg·L^{-1}
- LPS from *P. gingivalis* at 5, 20, and 20 μg·L^{-1}
- LPS from *E. coli* O111:B4 at 5, 20, and 20 μg·L^{-1}
- Combination of RgpA (20 μg·L^{-1}) and LPS *P. gingivalis* (20 μg·L^{-1})

Confocal Microscopy on PPP With LPS

Platelet poor plasma was exposed to LPS from *P. gingivalis* (n = 10; 10 ng·L^{-1}; 30 min) and clotted with thrombin (7 U·mL^{-1}) on a microscope slide to create a fibrin fiber clot. Exposed clots were compared to their matched naïve samples by visualizing intrinsic fluorescence on a Zeiss LSM 780 confocal microscope with a Plan-Apochromat 63x/1.4 oil DIC M27 objective. Clotted samples were excited by the 488 nm laser with emission detected between 508 and 570 nm and by the 561 nm laser with emission detected between 593 and 700 nm. These settings were chosen after scanning the samples with the hyperspectral mode of the confocal with each laser and determining the best emission

TABLE 1 | TEG® parameters [modified from (57)].

Thromboplastic parameters	Description
R: Reaction time (minutes)	Time of latency from start of test to initial fibrin formation (amplitude of 2 mm); i.e., initiation time
α angle: (slope between the traces represented by R-time at 2 mm and K-time at 20 mm) (degrees)	The angle measures the speed at which fibrin build up and cross linking takes place, hence assesses the rate of clot formation; i.e., thrombin burst
MA: Maximal amplitude (mm)	Maximum strength/stiffness of clot. Reflects the ultimate strength of the fibrin clot; i.e., overall stability of the clot
MRTG: Maximum rate of thrombus generation ($Dyn{\cdot}cm^{-2}{\cdot}s^{-1}$)	The maximum velocity of clot growth observed or maximum rate of thrombus generation using G, where G is the elastic modulus strength of the thrombus in $dynes{\cdot}cm^{-2}$
TMRTG: Time to maximum rate of thrombus generation (minutes)	The time interval observed before the maximum speed of the clot growth
TTG: Total thrombus generation ($Dyn{\cdot}cm^{-2}$)	The clot strength: the amount of total resistance (to movement of the cup and pin) generated during clot formation. This is the total area under the velocity curve during clot growth, representing the amount of clot strength generated during clot growth

TABLE 2 | Rheometry parameters.

Rheometry parameter	Description
$G'_{LVE\ (linear\ viscoelastic\ range)}$	Stability of the clot at rest—this means at equilibrium conditions. Elastic behavior (reversible deformation) of the clot
Elastic limit	Start of non-linear deformation. The clot cannot relax into its original state beyond this critical shear stress
Breakup stress	Shear stress needed to either break the clot apart or break it from the rheometer plate to which it adheres

range for autofluorescent signal in these samples. The area coverage of the autofluorescent signal in the confocal images was analyzed using ImageJ, with differences in the autofluorescent signal taken to reflect differences in the structure of the clot. Thresholding between 26 and 255 on the greyscale provided a consistent analysis of the images (3 images per clot). The percentage fluorescent area to total area of each image was compared between control and LPS-exposed groups.

Confocal Microscopy With Airyscan on Fluorescent Fibrinogen With LPS

Fluorescently labeled Alexa Fluor™ 488 purified fibrinogen (2 $mg{\cdot}mL^{-1}$) was used to evaluate anomalous clotting, upon the addition of *P. gingivalis* LPS to the fibrinogen (100 $ng{\cdot}L^{-1}$; 30 min). Samples were clotted with thrombin (7 $U{\cdot}mL^{-1}$) on a microscope slide and viewed with the Zeiss MP880 confocal microscope in Airyscan mode. Exposed clots were compared to their matched naïve samples by exciting the fibrin fibers with the 488 nm laser and collecting the emission with band pass filters 420–480 and 495–550 nm.

Confocal Microscopy on Fluorescent Fibrinogen With LPS

Fluorescently labeled Alexa Fluor™ 488 purified fibrinogen (2 $mg{\cdot}mL^{-1}$) was exposed to *E. coli* LPS (20 $ng{\cdot}L^{-1}$; 30 min) or *P. gingivalis* LPS (20 $ng{\cdot}L^{-1}$; 30 min). Naïve and LPS-exposed samples were clotted with thrombin (7 $U{\cdot}mL^{-1}$) on a microscope slide and viewed on a Zeiss LSM 780 confocal microscope with a Plan-Apochromat 63x/1.4 oil DIC M27 objective. Images were captured in lambda mode with the 488 nm laser and the GaAsP detector, which measures fluorescent emission between 410 and 695 nm across 32-channels, at 8.9 nm intervals. Multidimensional images were acquired as z-stacks and processed as maximum intensity projections in the ZEN software.

Correlative Atomic Force Microscopy and Raman Microspectroscopy on Fibrinogen With LPS

AFM-Raman was used to analyse the potential fiber structure changes in purified fibrinogen upon exposure to LPS from *P. gingivalis* (100 $ng{\cdot}L^{-1}$; 30 min). Purified fibrinogen was clotted on 10 mm gold-coated coverslips (HORIBA Scientific, France) with thrombin (7 $U{\cdot}mL^{-1}$). Naïve clots were prepared with the addition of thrombin. The glass coverslips were allowed to dry for about 2 min, before being submerged in PBS, followed by fixation in 4% formaldehyde and 1% osmium tetroxide, with PBS wash steps in between. Samples were dehydrated in increasing grades ethanol, before an ultimate HMDS drying step.

The characterization of the samples was performed with a LabRAM Nano. This multi-analysis platform consists of a Raman microspectrometer (LabRAM HR Evolution, HORIBA) combined with an AFM (SmartSPM, HORIBA Scientific) for chemical and physical analysis of the same samples area. The system is based on a reflection configuration capable of approaching the objective lens (Mitutoyo, 100× magnification, NA = 0.7, 20 mm working distance) from top illumination to the sample surface. Incident light is focused through the objective lens onto the apex of the AFM tip probe. In this study, micro-Raman images were measured with the 473 nm laser as the excitation source (3 mW maximum at the sample). Initially, three different wavelengths (473, 532, and 633 nm) were tested. It was determined that the 473 nm was the best choice, and the Raman spectrum was measured in one window. The LabRAM Nano is equipped with an Edge filter to cut the Rayleigh signal so that the Stokes signal could be measured. Raman images were collected from 10 μm^2 regions with 0.3 μm pixel steps. Acquisition time of each Raman spectrum is 30 s (one spectrum/image pixel). Correlated AFM images were obtained in AC mode using an ACCESS-NC Silicon probe (k = 25–95 N/m, f = 200–400 kHz, AppNano, US). The shape of the probes allows a direct visualization of the tip apex, which permits correlation with the excitation Raman. AFM images were acquired from 20

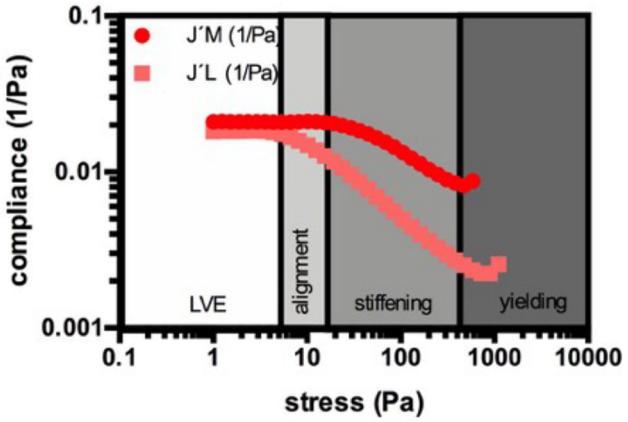


FIGURE 1 | Example for the suggested regions during the strain response of a naïve PDP clot. The minimum-strain compliance shown here (J'_M) reflects the tangent modulus at zero instantaneous stress, whereas the large-strain compliance (J'_L) reflects the secant modulus at maximum stress. During the linear viscoelastic behavior (LVE) the clot is in its equilibrium and the compliances merge and remain constant. With increasing stress the compliances start to diverge, which marks the elastic limit and the onset of the non-linear behavior. Now, the network elongates depending on its individual architecture. This period is characterized by progressive alignment of singular structures in the clot to establish a new architecture in which all structures are stretched out (here the microscopical changes in the clot take place). As soon as both compliances decrease together, the phase of shear-stiffening starts. The stretched network elongates as a whole and stiffens when the loads increase. When the stress becomes too high, the clot breaks either abruptly or yields. Yielding includes breaking of branch points or singular fibers in the network.

× 10 μm areas (300 × 150 pts) for the control sample and 20 × 20 μm areas (300 × 300 pts) for the experimental sample.

Viscoelastic Analysis

The Thrombelastograph® (TEG®) 5000 Hemostasis Analyzer (Haemoscope Corp) was used to measure the viscoelastic properties of blood, with the measured parameters listed in **Table 1**. PPP samples were exposed to LPS from *P. gingivalis* ($n = 10$; 10 ng·L^{-1}) or RgpA ($n = 30$; 500 ng·L^{-1}) for 30 min, with exposed samples compared to their matched naïve samples. (Initially we also exposed samples for 1 h, however, a longer exposure time did not significantly change the TEG® results). Prepared PPP was placed in a TEG® cup, together with 0.01 M calcium chloride ($CaCl_2$) to activate the coagulation process. The process was allowed to run until maximal amplitude (MA) was reached.

Rheometry of WB and PDP With LPS and RgpA

Whole blood (WB) ($n = 2$) and PDP ($n = 2$) were subjected to rheometry analysis on a Physica MCR 301 rheometer (Anton Paar, Austria) equipped with a Peltier controlled stainless steel sand-blasted cone-plate system (diameter 50 mm), mounted by a tempered hood and an evaporation blocker filled with silicon oil. The Rheocompass™ software (v1.22, Anton Paar, Austria) was used for data acquisition.

Samples were prepared by exposing blood from control donors for 1 h to either (1) LPS from *E. coli* (20 ng·L^{-1}), (2) LPS from *P. gingivalis* (20 or 20 μg·L^{-1}), or (3) RgpA (100 or 250 ng·L^{-1}). Matching control runs were diluted with the same volume of vehicle as for the exposed samples. Experiments were run in technical triplicate.

Whole blood and plasma were clotted by addition of 0.01 M $CaCl_2$ and clots were generated in the cone-plate geometry. A constant sinusoidal strain amplitude (0.1%, 1.5 Hz) was set to observe the process of clot formation with minimal interference. These time sweeps were conducted until a G' plateau was reached, at which point an amplitude sweep test was started. The amplitude sweep tests were stress-controlled with a logarithmic ramp from 1 to 5,000 Pa at constant angular frequency ($\omega = 1$ rad s^{-1}).

The rheometry parameters discussed in this paper are given in **Table 2**. During the amplitude sweep tests, we continuously monitored the resulting strain ($\gamma(\omega)$) of the material, which is the response of the clot to the applied sinusoidal stress ($\tau(\omega)$). The shift of the phase angle (δ) allows the calculation of the storage modulus (G') by multiplying the stress-strain relationship ($\tau(\omega)/\gamma(\omega)$) with cos(δ). G' serves as a measure of the reversibly stored and thus recoverable deformation energy and represents clot stiffness. As long as G' is maintained while the shear stress increases, the clot remains in its linear viscoelastic range (in its equilibrium) and experiences only elastic deformation. The clot can return into its initial form when the sinusoidal stress input crosses the 0-point. This can be also seen in the output waveform signal, which remains sinusoidal. With the continuous increase of shear force, a deviation from the initial G' value and a change in the output waveform signal occurs, which marks the onset of the non-linear response. From this shear stress onwards, the clot cannot return into its initial equilibrium state since the stronger deformation does not allow full recovery. The borderline between the linear and the non-linear behavior marks the elastic limit of the clot. As stresses become higher, non-linearity increases until the clot breaks. Since G' can be a misleading measure of the elastic modulus of plastically deforming clots, because other harmonic components may also store energy [see (58)], we applied the model of Ewoldt et al. (59), which is integrated in the Rheocompass software, to calculate clot compliance out of Bowditch-Lissajous plots using an approach that is geometrically motivated (60). The minimum-strain compliance shown here (J'_M) reflects the tangent modulus at zero instantaneous stress, whereas the large-strain compliance (J'_L) reflects the secant modulus at maximum stress (**Figure 1**). At equilibrium (linear clot behavior), both compliances merge, whereas out of equilibrium they diverge (non-linear behavior). Certain points on the curves indicate certain processes in the network, e.g., fiber bending and stretching out network inhomogeneities at intermediate shear stresses, stretching of the clot as a whole in shear direction at higher shear stresses, and weakening or even breaking of network points prior to complete breakup at highest-most shear stresses. **Figure 1** shows these suggested regions. We propose that not only an

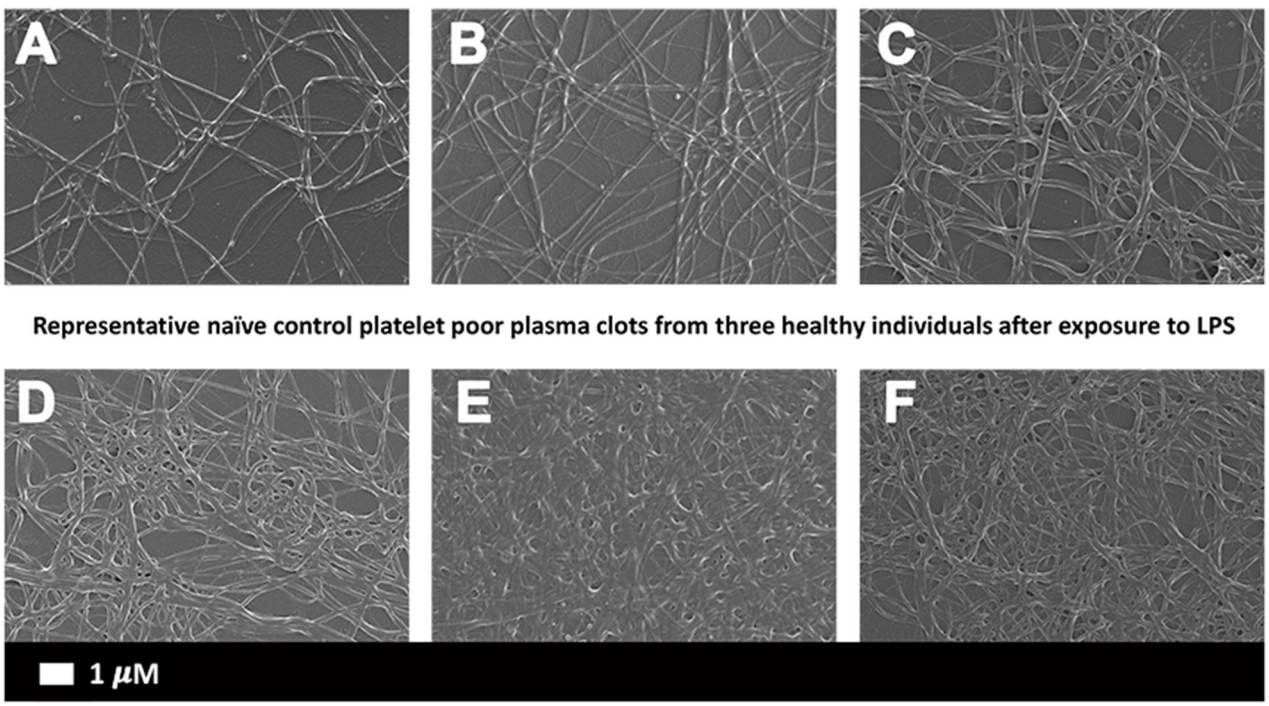


FIGURE 2 | Scanning electron micrographs of **(A–C)** representative naïve control plasma clots and **(D–F)** matched clots with added LPS from *P. gingivalis* (Scale bar: 1 μm).

upshift or downshift of the curves—indicating higher or lower compliances—must be considered to classify clots, but also changes in the shape of the compliance curves as they indicate specific clot behaviors. For example, the stress needed to fully stretch out the clot as a whole indicates the end of microscopic processes within the fiber network. Only if all branch points and inhomogeneities are aligned to the force lines, the clot stretches as a whole, which is referred to as macroscopic shear stiffening.

Statistical Analysis

Statistical analyses were performed on GraphPad Prism 7.04 with values of significance stated at $p < 0.05$. All data were subjected to Shapiro-Wilks normality tests. A paired *T*-test was performed on parametric data with the data expressed as mean ± standard deviation, whereas the Mann-Whitney *U*-test was used on unpaired non-parametric data and the Wilcoxon matched-pairs signed rank test was used on non-parametric data that was paired with the data expressed as median [Q1–Q3] (all two-tailed).

RESULTS

Scanning Electron Microscopy of PPP Clots With LPS

The differences in PPP clot ultrastructure in naïve clots (**Figures 2A–C**) and in the presence of *P. gingivalis* LPS (**Figures 2D–F**) were evaluated. Statistical analysis of fibrin fiber thickness showed a significant ($p < 0.0001$) increase in fiber width between naïve [0.19 μm (0.14–0.25)] and LPS-exposed [0.27 μm (0.2–0.37)] samples.

Scanning Electron Microscopy of Purified Fibrinogen Clots With LPS and RgpA

The effect of LPS from *P. gingivalis* and *E. coli* as well as RgpA on the network structure of pure fibrin fiber networks was examined by SEM (**Figure 3**). Addition of either LPS from *E. coli* or *P. gingivalis* resulted in greater observations of fused and thicker fibers. Fiber width was statistically greater ($p < 0.0001$) in clots exposed to LPS from *P. gingivalis* [0.22 μm (0.17–0.33)] compared to naïve clots [0.18 μm (0.13–0.22)]. Previously, we reported the same changes in fiber thickness for LPS form *E. coli* (2). Exposure to RgpA led to various changes in the network architecture of fibrin clots. Most fibers were observed as looser networks of clumped fibers, sporadically distributed throughout the SEM preparation and with disruptions to the fiber structure. In the few areas of confluent fibers, some breaks and disruptions to the network could be noted. The combination of RgpA and LPS from *P. gingivalis* seemed to cause breaks and disruptions in the regular fiber networks.

Confocal Microscopy of PPP Clots With LPS

Figure 4 shows representative micrographs of the autofluorescence signal in control and LPS-exposed clots

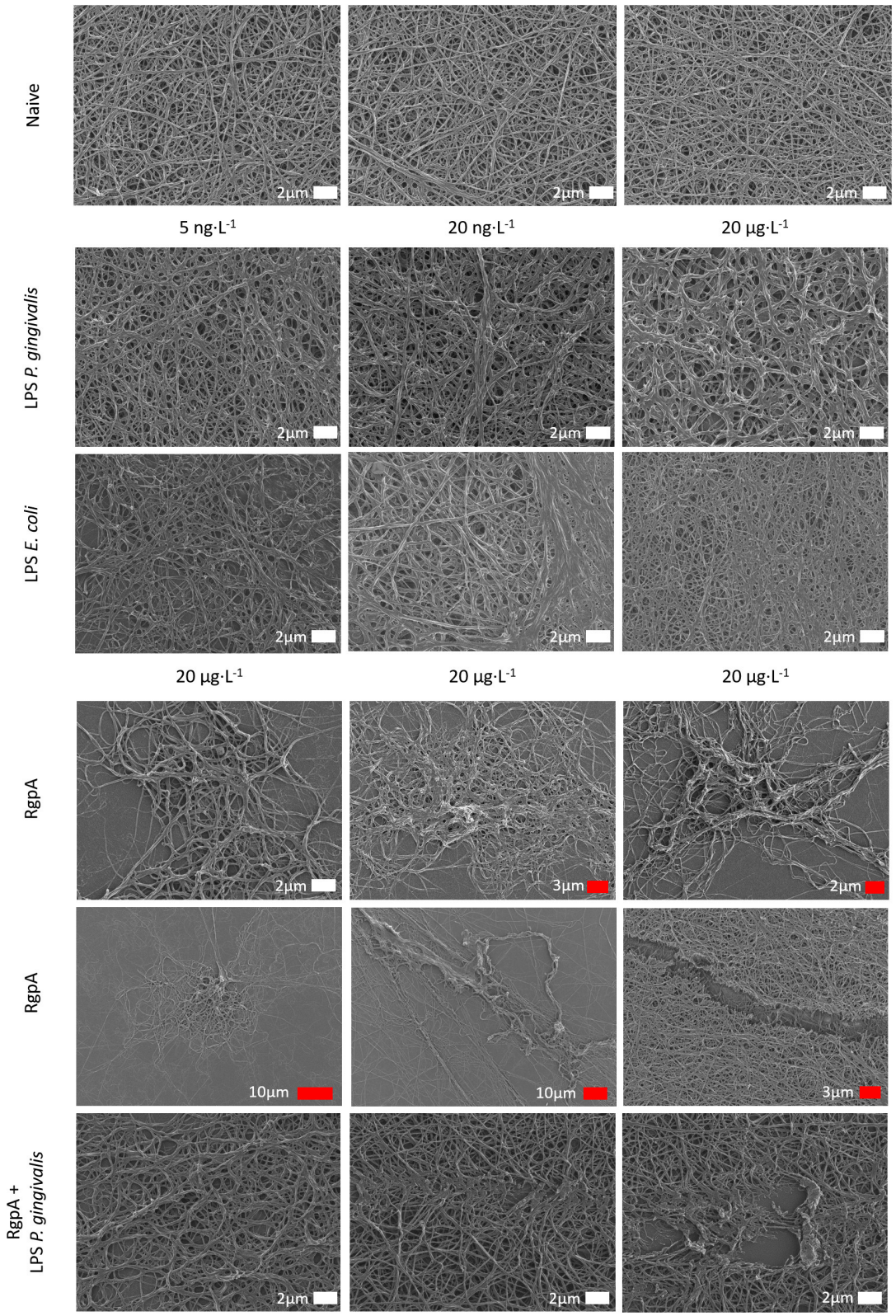


FIGURE 3 | Scanning electron micrographs of purified fibrinogen clots exposed to bacterial products at varying concentrations (white scale bars indicate identical scales).

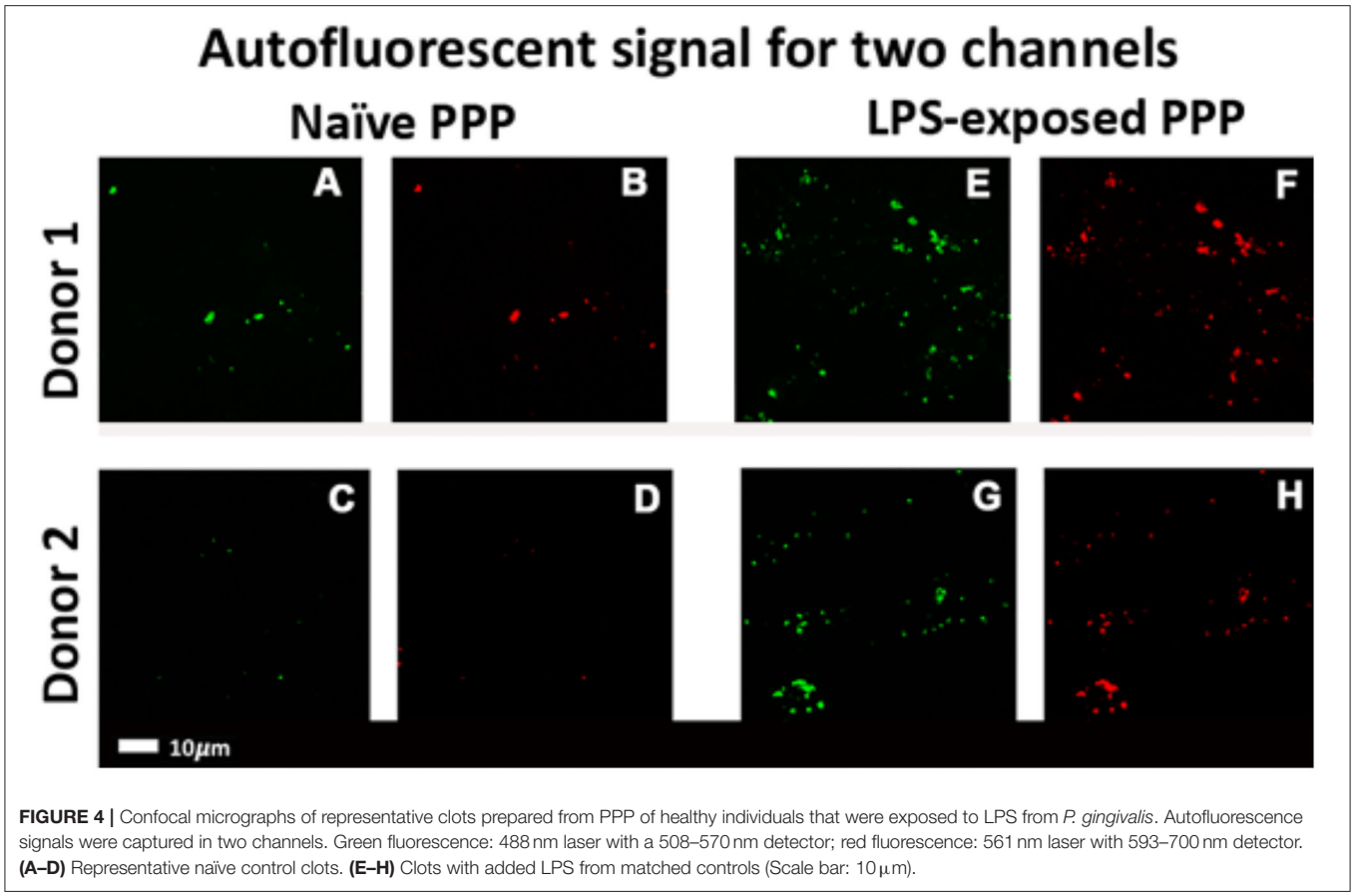


FIGURE 4 | Confocal micrographs of representative clots prepared from PPP of healthy individuals that were exposed to LPS from *P. gingivalis*. Autofluorescence signals were captured in two channels. Green fluorescence: 488 nm laser with a 508–570 nm detector; red fluorescence: 561 nm laser with 593–700 nm detector. **(A–D)** Representative naïve control clots. **(E–H)** Clots with added LPS from matched controls (Scale bar: 10 μm).

for the 488 and 561 nm lasers. The total autofluorescent area of the clots after LPS exposure [1.01% (0.8–1.4)] was significantly ($p < 0.001$) increased compare to the control [0.16% (0.067–0.31)]. Changes in the intrinsic optical properties of fibrinogen might reflect changes to fibri(ogen) (61).

Confocal Microscopy on Fluorescent Fibrin(Ogen) Clots With LPS

We also investigated protein misfolding in fluorescent fibrinogen using Airyscan technology (Zeiss MP880), after addition of LPS from *P. gingivalis*. The control fibrin(ogen) clot (**Figure 5A**) showed typical netted fibrin fibers, whereas the LPS-exposed samples (**Figures 5B,C**) show areas of intense fluorescence and have a more densely formed fibrin network. Additionally, confocal z-stacks (Zeiss LSM 780) of fibrinogen exposed to *E. coli* and *P. gingivalis* also illustrated changes in the fibrinogen network structure. The control clots (**Figure 5D**) showed loose networks of fibers, whereas LPS-exposure (**Figures 5E,F**) show much denser fibers networks, a feature of hypercoagulation.

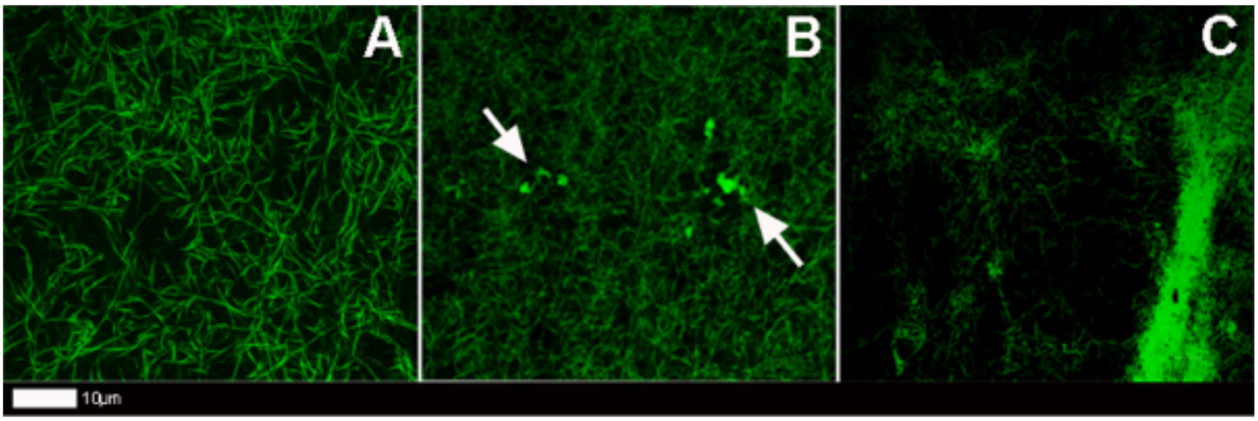
Correlative Atomic Force Microscopy and Raman Microspectroscopy on Fibrinogen With LPS

Correlative AFM and Raman images were obtained from naïve and LPS-exposed samples (**Figure 6**). The amide I intensity Raman band monitoring (**Figures 6B,D**) showed a higher Raman signal intensity on the fibers and perfect correlation with the AFM topography. The comparison between the average spectra from the two samples (after C-H stretching band intensity normalization) highlights some slight differences as band broadening, band position shift, and intensity ratio changes, which could indicate a possible ß-sheet unfolding in the LPS-exposed samples (**Figure 6E**).

Thromboelastography® of PPP With LPS and RgpA

Table 3 shows the TEG® results for PPP exposed to LPS from *P. gingivalis*, compared to matched naïve samples. Significant changes are seen in the *R*-value, α angle and TMRTG values, indicating accelerated clot formation and fiber cross-linking. This suggests that LPS-exposed clots forms faster, which is a feature of hypercoagulability.

The effect exerted by the RgpA protease on viscoelastic parameters of clotting is shown in **Table 4**. All six parameters assessed exhibited significant changes. RgpA pre-treatment shifts the coagulability to a more hypocoaguable state in terms of clotting time, which is represented by the three time-dependent parameters *R*-value (**), MRTG (***), and TMRTG (**), which are all increased compared to



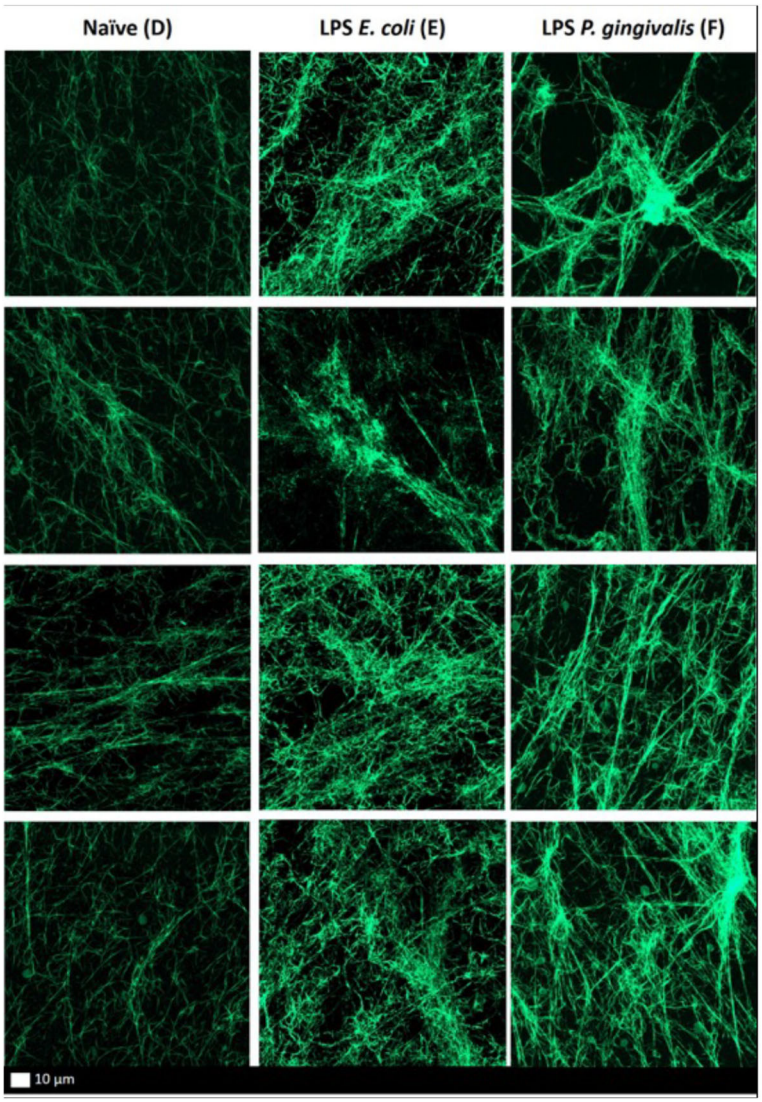


FIGURE 5 | (A–C) Airyscan micrographs of fluorescent fibrinogen. **(A)** Naïve clot showing a normal distribution of fibrin fibers. **(B,C)** LPS-exposed (*P. gingivalis*) fluorescent fibrinogen, where plaque-type areas are present (white arrows) (Scale bar: 10 µm). **(D–F)** Confocal lambda maximal intensity projections of fluorescent fibrinogen. Each column shows four representative projections per exposure. **(D)** Naïve clot. **(E)** LPS-exposed (*E. coli*) clot. **(F)** LPS-exposed (*P. gingivalis*) clot.

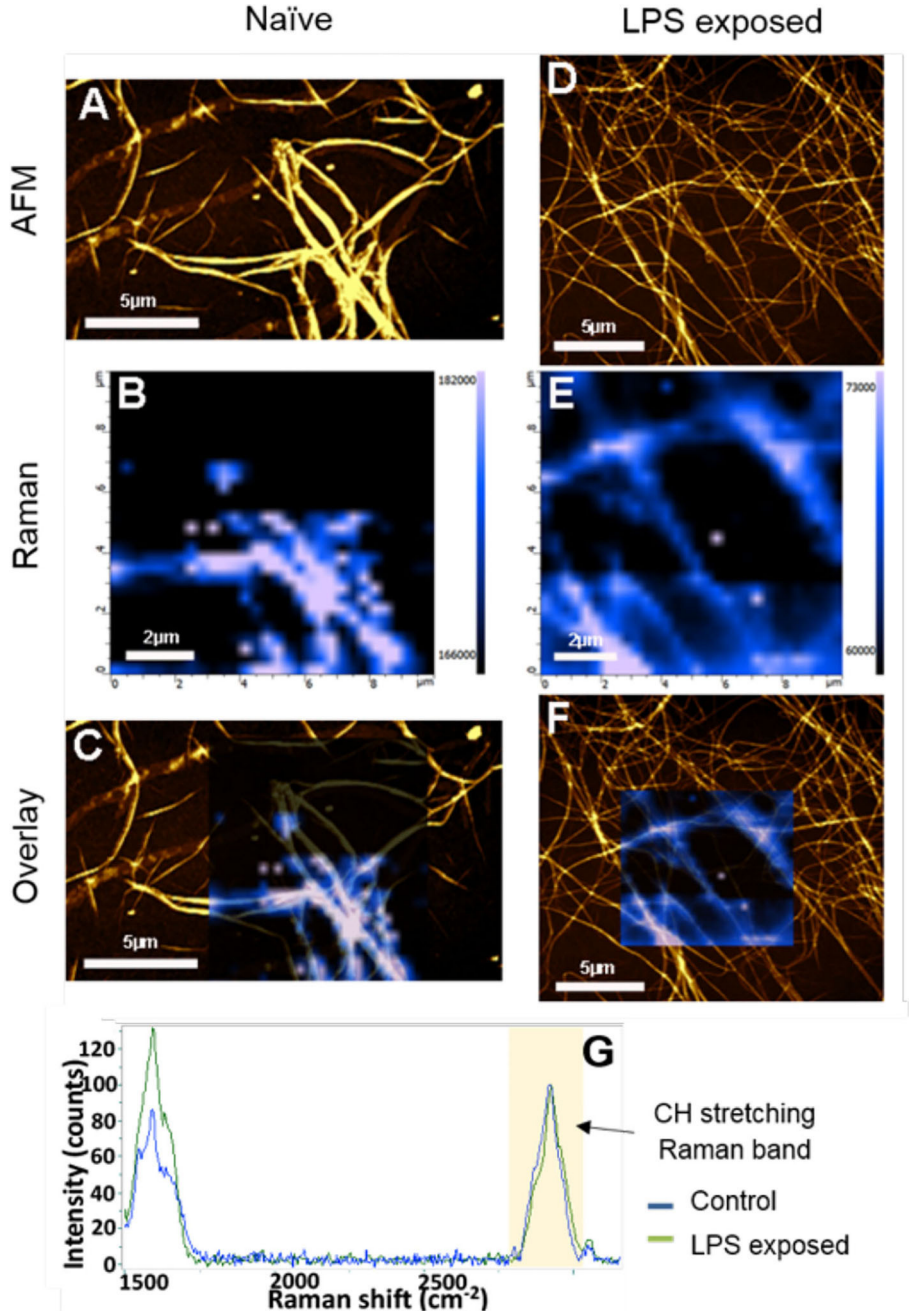


FIGURE 6 | AFM, Raman, and AFM-Raman correlative images of the **(A–C)** control and **(D–F)** *P. gingivalis* LPS-exposed fibrinogen, as well as **(G)** the average Raman spectra of control sample (blue Raman spectrum) and LPS-exposed sample spectra (green Raman spectrum) The Raman images are the Amide I intensity maps of naïve clots vs. LPS-exposed clots. They are overlaid to the AFM images **(B,D)**. Blue spectrum is average spectrum of a Raman map of the naïve sample. Green spectrum is average spectrum of a Raman map of LPS-exposed sample [Scale bars: **(A,C,D,F)**: 5 µm; **(B,E)**: 2 µm].

controls. The lower α-angle (**) reflects that the fibrin build-up is slower in the exposed samples, resulting in a reduction of fibrin cross-linking. In addition, the resultant clot strength and stability measured by MA (*) was increased in the RgpA group, whereas strength measured by TTG (*) was decreased.

Rheometry of Whole Blood (WB) and Platelet Depleted Plasma (PDP) With LPS and RgpA

Rheometry results for exposed samples and their matched control runs are recorded in **Table 5** and **Figure 7**, that shows minimum (J'_M) and large (J'_L) strain compliance graphs of naïve vs. exposed

TABLE 3 | TEG® results of naïve and *P. gingivalis* LPS-exposed control PPP.

Parameter	Control	LPS	*p*-value
R	13.80 ± 2.73	10.04 ± 2.73	0.02 (*)
α angle	55.61 ± 5.33	60.28 ± 3.16	0.04 (*)
MA	22.75 ± 3.36	21.05 ± 4.94	0.3
MRTG	2.73 ± 0.84	3.28 ± 0.76	0.09
TMRTG	15.74 ± 3.26	10.77 ± 2.16	0.007 (**)
TTG	148.61 ± 28.36	135.22 ± 40.48	0.3

*Data are represented as mean ± standard deviation. Statistical significance was established at p<0.05 (*p < 0.05; **p < 0.01).*

TABLE 4 | TEG® results of naïve control and RgpA-exposed PPP.

Parameter	Control	RgpA	*p*-value
R	9.15 [7.8–11.8]	11.5 [8.13–13.53]	0.0011 (**)
α angle	66 [59.73–68.95]	59.63 ± 8.89	0.0014 (**)
MA	24.57 ± 6.21	23.45 [18.5–25.15]	0.021 (*)
MRTG	4.2 ± 1.72	3.07 [2.17–4.56]	0.0001 (***)
TMRTG	10.42 [8.9–13.38]	13.75 [8.96–17.19]	0.0032 (**)
TTG	167.7 ± 56.79	149.8 ± 43.28	0.022 (*)

*Data are represented as either mean ± standard deviation or median [Q1–Q3]. Statistical significance was established at p < 0.05 (*p < 0.05; **p < 0.01; ***p < 0.001).*

samples, which reflect the behavior of clots at cyclic stress loading of clots during amplitude sweep tests. In PDP, all the different treatments increased the median linear elastic shear modulus of the clot, and led the non-linear response to start at similar and lower stress when compared to matched controls. However, the overlapping confidence intervals suggest a very small influence of the exposures on the G' modulus. Only exposure with LPS from *P. gingivalis* could increase the breakup stress.

Additional information can be gained from the compliances. LPS-exposure reduced both compliances (**Figure 7C**). They were also maintained over a larger shear stress range compared to their matched controls. Our model described in **Figure 1** suggests that network alignment must be prolonged, which allows macroscopic shear-stiffening to start at higher stresses. The arrows in **Figure 7C** show this critical shear stress. Only after a shear stress of 380 Pa did the confidence intervals (CI) of the large-strain compliances (J'_L) of LPS-clots and control clots overlap, indicating similar behavior of fully stretched clots after that. In RgpA-exposed PDP samples the compliances varied substantially at the start of the stress test (see also the high CI of the median G'_{LVE} value in **Table 5**), indicating that different network architectures have formed. During the stress tests, the compliances of the exposed and non-exposed samples converge completely (**Figure 7A**) indicating similar behavior of fully stretched clots.

In WB, the moduli were much higher than in PDP, but the RBCs blunted many effects that were seen in PDP, e.g., there was almost no shear-stiffening. Rather, WB clots showed a pronounced phase of shear-softening prior to the onset of weak shear-stiffening. In other words, the compliances increased until higher shear stress before they dropped. Other microscopic and macroscopic processes will take place in a stressed clot when blood cells are present. WB clot exposure to both high and low concentrations of *P. gingivalis* LPS resulted in a decrease of clot stiffness (which was more pronounced with the higher concentration), however, breakup stress was unaffected in both exposures. RgpA-exposed WB samples appeared to be stiffer when in near-equilibrium condition (see the median G'_{LVE} and also its high CI similar to PDP in **Table 5**) compared to their matched controls but they broke earlier while they were still in the phase of softening. It appears that RgpA exposure prevented shear-stiffening (**Figure 7B**).

DISCUSSION

Bacterial inflammagens in circulation can influence coagulation parameters that may result in abnormal clot formation and structure. Here we present the effects of gingipain R1 and LPS from *P. gingivalis* and *E. coli* on clot morphology and mechanics of clots produced from PPP, PDP, WB, as well as purified fibrinogen models. We point out that Jain et al. (56) reported that some LPS preparations might have lipoprotein contaminants present. Our LPS results on blood clots may therefore be due to LPS and/or the associated lipoprotein that might be present in the purchased LPS form Sigma. First looking at the morphology of LPS-exposed samples, SEM analysis in all our clot models showed that LPS from both *P. gingivalis* and *E. coli* caused the clot to become more dense and confluent in nature (**Figures 2**, **3**). Individual, elongated fibers are visible in the controls, but in the exposures, the fibers are arranged to become dense and netlike (see **Figure 2**). This is in line with our previous analysis where LPS from *E. coli* caused fibers to become more netlike (in both PPP and fibrinogen models) (2). Our confocal and airyscan analyses on fluorescent fibrinogen (**Figure 5**) support the SEM observations. We also investigated the development of anomalous protein structures in PPP using autofluorescent signal when the LPS from *P. gingivalis* was added. Here, a significantly increased autofluorescent signal was assessed by the area analysis (**Figure 4**). These differences in fluorescent signal might reflect a structural change in the protein packaging in the presence of thrombin (61). Correlative AFM and Raman images from controls and *P. gingivalis* LPS-exposed samples (**Figure 6**), shows slight differences as band broadening, band position shift, and intensity ratio changes. Although the differences are small, they indicate a change in the local symmetry of the fibrinogen molecule in the C-H areas, localized on the fibers in the LPS-exposed samples (**Figure 6E**). This supports the altered morphological appearance of the clots as seen with SEM. This is the first report that shows LPS from *P. gingivalis* may chemically modify the structure of the fibrin(ogen) clot. These modifications may possibly be related to molecular changes of the fibrin(ogen) protein itself. Previously, we reported that LPS from *E. coli* added to fibrin(ogen) and plasma, can undergo structural changes that might be amyloid in nature. This was confirmed using the fluorescent amyloid stains, thioflavin T, and Amytrackers (52).

TABLE 5 | Rheometry data.

Sample	G'_{LVE} [Pa]	Elastic limit [Pa]	Breakup stress [Pa]
Whole blood (WB)			
Control 1	174.1 [156.2–192.0]	4.4 [4.4–4.4]	442 [305–578]
Control 1 + RgpA (100 ng·L^{-1})	201.9 [118.7–222.2]	4.5 [1.9–5.5]	341 [247–467]
Control 2	324.0 [291.5–332.1]	1.2 [1.2–1.2]	1,377 [1,372–1,705]
Control 2 + PG LPS (20 ng·L^{-1})	297.3 [261.7–314.1]	1.2 [1.2–1.5]	1,380 [1,376–1,702]
Control 2 + PG LPS (20 μg·L^{-1})	265.6 [211.8–284.6]	1.2 [1.2–1.9]	1,376 [1,374–1,377]
Platelet depleted plasma (PDP)			
Control 1	40.36 [38.58–42.71]	2.9 [2.9–2.9]	1,113 [1,109–1,370]
Control 1 + RgpA (250 ng·L^{-1})	45.52 [37.75–52.75]	2.3 [2.3–2.9]	1,110 [896–1,112]
Control 3	47.26 [44.56–54.78]	2.9 [1.9–5.4]	1,112 [584–1,113]
Control 3 + EC LPS (20 ng·L^{-1})	72.30 [61.77–76.84]	2.9 [2.9–3.6]	1,113 [898–1,116]
Control 3 + PG LPS (20 ng·L^{-1})	68.05 [67.60–77.92]	3.6 [2.9–3.6]	1,378 [1,112–1,379]

Data presented as median [lower and upper confidence interval] of the triplicate runs. EC, E. coli; PG, P. gingivalis.

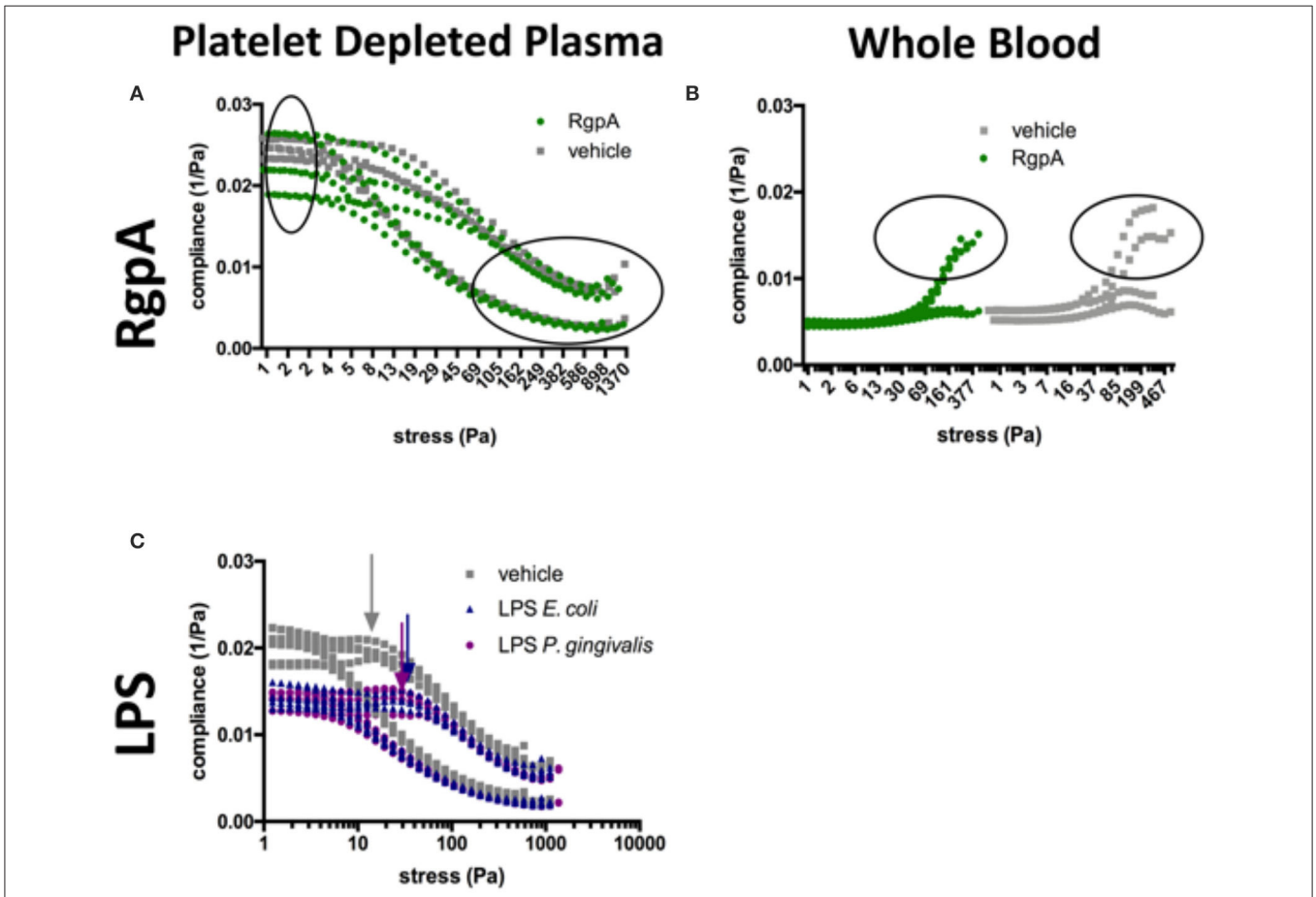


FIGURE 7 | Compliance graphs of naïve vs. exposed samples, which reflect the intra-cycle behavior of clots during amplitude sweep tests. **(A)** RgpA-exposed PDP, **(B)** RgpA-exposed WB, **(C)** LPS-exposed PDP (*E. coli* and *P. gingivalis*). Graphs plot the sample runs in triplicate (PDP) and duplicate (WB).

When RgpA is added to our different models, clots (viewed with SEM) were not confluent, but rather appeared mostly in sporadic clumps with masses of higher density surrounded by less dense areas. The fibers that did form showed a sparse and heterogeneous structure (see **Figure 3**). This was also previously established with confocal microscopy, where we added RgpA to fluorescent fibrin(ogen) and noted a decrease in formation of fibrin(ogen) networks (54). We propose that such heterogeneous

clots will not be able to transmit hydrodynamic forces applied to them uniformly through the entire formed clot, but rather along their most elastic structures, while other parts of the clot remain mostly unstressed. This could pose excessive stress to existing structures, which could be a risk factor for clot pathologies. Our mechanical stress tests support this hypothesis (**Figure 7B**).

We further studied clot forming kinetics by 6 different parameters obtained from our TEG® tests and the mechanical response of our various clot models, before and after addition of LPS from *E. coli* and *P. gingivalis* as well as RgpA, by using rheometry. See **Tables 1**, **2** for the various parameters of TEG® and rheometry. After generating the WB and PDP clots in the cone-plate geometry of the rheometer we submitted them to increasing sinusoidal shear stresses and probed their strain responses. We also applied a model to differentiate the phase of network orientation at intermediate shear stresses from the subsequent phase of whole network stretch at higher shear forces.

RgpA-exposed clots seem to break abruptly at lower stresses in our WB model, whereas naïve clots showed a gradual yielding until the clot breaks at higher shear stresses (compare the encircled regions in **Figure 7B**). The same trend is seen in our PDP model, however, in this model breakup is not as abrupt as in the WB model. The heterogeneous structure seen in **Figure 5** is also reflected by the high confidence interval of the linear elastic shear modulus and the compliances near equilibrium, however, the ability to shear-stiffen is unaffected (see **Figure 7A**). Our TEG® results showed that RgpA causes the PPP clot to form significantly slower (indicated by the *R*-value), and with a general alteration in clot strength (indicated by the MA and the TTG value). These results are consistent with the expectation that RgpA is a proteolytic enzyme. This is consistent with previous papers that looked at the proteolytic actions of RgpA on fibrinogen structure (48, 62). Our TEG® results also suggest that in the presence of LPS from *P. gingivalis*, the clot forms faster (R and TMRTG parameters), but the clot stiffness is not affected (MA and TTG). This is consistent with our previous results using LPS from *E. coli* (2, 52). These results are consistent with the finding of the rheometry by looking at the clot stiffness at its equilibrium (G'_{LVE}). When we analyse our rheometry results further, in the PDP samples where we added the two LPSs the compliances were not only lower until 380 Pa applied shear stress, but shear-stiffening started also at higher stresses. This suggests that LPS-exposed clots will need more shear stress to stretch out all inhomogeneities before they can stretch like the control sample can (see **Figure 7C**, the arrows indicate this drop in the compliance, showing the shear stress where shear-stiffening begins). Such inhomogeneities are seen in our SEM samples as a denser and less uniform clot structure. Shear stiffening is a common property of biological fibers (63) and is *per se* not affected by LPS in our models. However, it is obvious that processes that soften the clot, such as fiber buckling and bending and network alignment, compete with processes that stretch the network and therefore shift the onset of macroscopic shear-stiffening to higher stresses.

CONCLUSIONS

In this paper we bring together evidence that bacterial LPSs and RgpA can affect both clot structure and mechanics. This has significant implications for clotting and clot formation when these inflammagens enter into circulation, via various routes. These routes may include the gut when dysbiosis is present (leaky gut), the urinary tract (during infections), as well as the mouth area, during gingivitis and periodontitis. It is well-known that these entry pathways are active in most inflammatory conditions. When in circulation, these inflammagens interacts with soluble fibrinogen, where they bring about all the effects we have described (mechanical and structural changes). In future, it would also be valuable to investigate the effects of Kgp, and RgpB, and combinations of the molecules with RgpA, as all of these molecules contribute significantly to the virulence of the bacterium (64, 65). Ultimately, these interactions are associated with systemic inflammation and coagulation pathologies. The magnitude of this effect differs in plasma and purified fibrinogen and most likely exists due to the presence of inhibitory and target molecules in plasma such as albumin and other proteins. The presence of these inflammagens in the circulation of individuals with various cardiovascular and systemic inflammation conditions, including T2DM, may have far-reaching healthy effects on blood clotting.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. The datasets generated as well as figure micrographs analyzed during the current study are available: https://1drv.ms/u/s!AgoCOmY3bkKHioRESgGKZsHuntFsoA?e=BTUXvr.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Stellenbosch University Human Ethics Committee. The patients/participants provided their written informed consent to participate in this study. Ethics was also obtained from the Ethics Committee of the Medical University Vienna, Austria.

AUTHOR CONTRIBUTIONS

JN: TEG®, rheometry, and SEM. TF: TEG® and SEM. MP: rheometry, SEM, confocal, and data analysis. CV: technical assistance. UW: rheometry. EP: study leader. All authors approved submission of the paper. DK: editing of paper and co-corresponding author. OL: raman analysis.

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REFERENCES

1. Pretorius E, Bester J, Kell DB. A bacterial component to Alzheimer's-type dementia seen via a systems biology approach that links iron dysregulation and inflammagen shedding to disease. *J Alzheimer's Dis.* (2016) 53:1237–56. doi: 10.3233/JAD-160318
2. Pretorius E, Mbotwe S, Bester J, Robinson CJ, Kell DB. Acute induction of anomalous and amyloidogenic blood clotting by molecular amplification of highly substoichiometric levels of bacterial lipopolysaccharide. *J R Soc Interface.* (2016) 13:20160539. doi: 10.1101/053538
3. Pretorius E, Mbotwe S, Kell DB. Lipopolysaccharide-binding protein (LBP) reverses the amyloid state of fibrin seen in plasma of type 2 diabetics with cardiovascular co-morbidities. *Sci Rep.* (2017) 7:9680. doi: 10.1038/s41598-017-09860-4
4. Kell DB, Pretorius E. Proteins behaving badly. substoichiometric molecular control and amplification of the initiation and nature of amyloid fibril formation: lessons from and for blood clotting. *Prog Biophys Mol Biol.* (2017) 123:16–41. doi: 10.1016/j.pbiomolbio.2016.08.006
5. Kell DB, Pretorius E. No effects without causes: the iron dysregulation and dormant microbes hypothesis for chronic, inflammatory diseases. *Biol Rev Camb Philos Soc.* (2018) 93:1518–57. doi: 10.1111/brv.12407
6. Potgieter M, Bester J, Kell DB, Pretorius E. The dormant blood microbiome in chronic, inflammatory diseases. *FEMS Microbiol Rev.* (2015) 39:567–91. doi: 10.1093/femsre/fuv013
7. de Kort S, Keszthelyi D, Masclee AA. Leaky gut and diabetes mellitus: what is the link? *Obesity Rev.* (2011) 12:449–58. doi: 10.1111/j.1467-789X.2010.00845.x
8. Kamer AR, Dasanayake A, Craig RG, Glodzik-Sobanska L, Bry M, de Leon MJ, et al. Alzheimer's disease and peripheral infections: the possible contribution from periodontal infections, model and hypothesis. *J Alzheimer's Dis.* (2008) 13:437–49. doi: 10.3233/JAD-2008-13408
9. Miklossy J. Emerging roles of pathogens in Alzheimer disease. *Expert Rev Mol Med.* (2011) 13:e30. doi: 10.1017/S1462399411002006
10. Cestari JA, Fabri GM, Kalil J, Nitrini R, Jacob-Filho W, de Siqueira JT, et al. Oral infections and cytokine levels in patients with Alzheimer's disease and mild cognitive impairment compared with controls. *J Alzheimer's Dis.* (2016) 52:1479–85. doi: 10.3233/JAD-160212
11. Gaur S, Agnihotri R. Alzheimer's disease and chronic periodontitis: is there an association? *Geriatr Gerontol Int.* (2015) 15:391–404. doi: 10.1111/ggi.12425
12. Holmstrup P, Damgaard C, Olsen I, Klinge B, Flyvbjerg A, Nielsen CH, et al. Comorbidity of periodontal disease: two sides of the same coin? An introduction for the clinician. *J Oral Microbiol.* (2017) 9:1332710. doi: 10.1080/20002297.2017.1332710
13. Shaik MM, Ahmad S, Gan SH, Abuzenadah AM, Ahmad E, Tabrez S, et al. How do periodontal infections affect the onset and progression of Alzheimer's disease? *CNS & Neurol Disord Drug Targets.* (2014) 13:460–6. doi: 10.2174/18715273113126660152
14. Sparks Stein P, Steffen MJ, Smith C, Jicha G, Ebersole JL, Abner E, et al. Serum antibodies to periodontal pathogens are a risk factor for Alzheimer's disease. *Alzheimers Dement.* (2012) 8:196–203. doi: 10.1016/j.jalz.2011.04.006
15. Kohler CA, Maes M, Slyepchenko A, Berk M, Solmi M, Lanctot KL, et al. The gut-brain axis, including the microbiome, leaky gut and bacterial translocation: mechanisms and pathophysiological role in Alzheimer's disease. *Curr Pharm Des.* (2016) 22:6152–166. doi: 10.2174/1381612822666160907093807
16. Kaur T, Uppoor A, Naik D. Parkinson's disease and periodontitis - the missing link? A review. *Gerodontology.* (2016) 33:434–8. doi: 10.1111/ger.12188
17. Hu X, Wang T, Jin F. Alzheimer's disease and gut microbiota. *Sci China Life Sci.* (2016) 59:1006–23. doi: 10.1007/s11427-016-5083-9
18. Fasano A, Bove F, Gabrielli M, Petracca M, Zocco MA, Ragazzoni E, et al. The role of small intestinal bacterial overgrowth in Parkinson's disease. *Mov Disord.* (2013) 28:1241–9. doi: 10.1002/mds.25522
19. Scheperjans F, Aho V, Pereira PA, Koskinen K, Paulin L, Pekkonen E, et al. Gut microbiota are related to Parkinson's disease and clinical phenotype. *Mov Disord.* (2015) 30:350–8. doi: 10.1002/mds.26069
20. Mulak A, Bonaz B. Brain-gut-microbiota axis in Parkinson's disease. *World J Gastroenterol.* (2015) 21:10609–20. doi: 10.3748/wjg.v21.i37.10609
21. Minato T, Maeda T, Fujisawa Y, Tsuji H, Nomoto K, Ohno K, et al. Progression of Parkinson's disease is associated with gut dysbiosis: two-year follow-up study. *PLoS ONE.* (2017) 12:e0187307. doi: 10.1371/journal.pone.0187307
22. Amar J, Serino M, Lange C, Chabo C, Iacovoni J, Mondot S, et al. Involvement of tissue bacteria in the onset of diabetes in humans: evidence for a concept. *Diabetologia.* (2011) 54:3055–61. doi: 10.1007/s00125-011-2329-8
23. Sato J, Kanazawa A, Ikeda F, Yoshihara T, Goto H, Abe H, et al. Gut dysbiosis and detection of "live gut bacteria" in blood of Japanese patients with type 2 diabetes. *Diabetes Care.* (2014) 37:2343–50. doi: 10.2337/dc13-2817
24. Slyepchenko A, Maes M, Machado-Vieira R, Anderson G, Solmi M, Sanz Y, et al. Intestinal dysbiosis, gut hyperpermeability and bacterial translocation: missing links between depression, obesity and Type 2 diabetes. *Curr Pharm Des.* (2016) 22:6087–106. doi: 10.2174/1381612822666160922165706
25. Poole S, Singhrao SK, Kesavalu L, Curtis MA, Crean S. Determining the presence of periodontopathic virulence factors in short-term postmortem Alzheimer's disease brain tissue. *J Alzheimer's Dis.* (2013) 36:665–77. doi: 10.3233/JAD-121918
26. Bester J, Soma P, Kell DB, Pretorius E. Viscoelastic and ultrastructural characteristics of whole blood and plasma in Alzheimer-type dementia, and the possible role of bacterial lipopolysaccharides (LPS). *Oncotarget.* (2015) 6:35284–303. doi: 10.18632/oncotarget.6074
27. Zhan X, Stamova B, Jin LW, DeCarli C, Phinney B, Sharp FR. Gram-negative bacterial molecules associate with Alzheimer disease pathology. *Neurology.* (2016) 87:2324–32. doi: 10.1212/WNL.0000000000003391
28. de Waal GM, Engelbrecht L, Davis T, de Villiers WJS, Kell DB, Pretorius E. Correlative light-electron microscopy detects lipopolysaccharide and its association with fibrin fibres in Parkinson's disease, Alzheimer's disease and Type 2 diabetes mellitus. *Sci Rep.* (2018) 8:16798. doi: 10.1038/s41598-018-35009-y
29. Creely SJ, McTernan PG, Kusminski CM, Fisher fM, Da Silva NF, Khanolkar M, et al. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am J Physiol Endocrinol Metab.* (2007) 292:E740–7. doi: 10.1152/ajpendo.00302.2006
30. Rempel JD, Packiasamy J, Dean HJ, McGavock J, Janke A, Collister M, et al. Preliminary analysis of immune activation in early onset type 2 diabetes. *Int J Circumpolar Health.* (2013) 72. doi: 10.3402/ijch.v72i0.21190
31. Kell DB, Pretorius E. To what extent are the terminal stages of sepsis, septic shock, systemic inflammatory response syndrome, and multiple organ dysfunction syndrome actually driven by a prion/amyloid form of fibrin? *Semin Thromb Hemost.* (2018) 44:224–38. doi: 10.1055/s-0037-1604108
32. Pretorius E, Akeredolu OO, Soma P, Kell DB. Major involvement of bacterial components in rheumatoid arthritis and its accompanying oxidative stress, systemic inflammation and hypercoagulability. *Exp Biol Med.* (2017) 242:355–73. doi: 10.1177/1535370216681549
33. Visser MJE, Kell DB, Pretorius E. Bacterial dysbiosis and translocation in psoriasis vulgaris. *Front Cell Infect Microbiol.* (2019) 9:7. doi: 10.3389/fcimb.2019.00007
34. Miyashita H, Honda T, Maekawa T, Takahashi N, Aoki Y, Nakajima T, et al. Relationship between serum antibody titres to Porphyromonas gingivalis and hs-CRP levels as inflammatory markers of periodontitis. *Arch Oral Biol.* (2012) 57:820–9. doi: 10.1016/j.archoralbio.2011.11.008

35. Liu Y, Zhang Y, Wang L, Guo Y, Xiao S. Prevalence of Porphyromonas gingivalis four rag locus genotypes in patients of orthodontic gingivitis and periodontitis. *PLoS ONE.* (2013) 8:e61028. doi: 10.1371/journal.pone.0061028
36. Shanker J, Setty P, Arvind P, Nair J, Bhasker D, Balakrishna G, et al. Relationship between periodontal disease, Porphyromonas gingivalis, peripheral vascular resistance markers and coronary artery disease in Asian Indians. *Thrombos Res.* (2013) 132:e8–14. doi: 10.1016/j.thromres.2013.04.023
37. Amar S, Engelke M. Periodontal innate immune mechanisms relevant to atherosclerosis. *Mol Oral Microbiol.* (2015) 30:171–85. doi: 10.1111/omi.12087
38. Singhrao SK, Harding A, Simmons T, Robinson S, Kesavalu L, Crean S. Oral inflammation, tooth loss, risk factors, and association with progression of Alzheimer's disease. *J Alzheimer's Dis.* (2014) 42:723–37. doi: 10.3233/JAD-140387
39. Olsen I, Singhrao SK. Is there a link between genetic defects in the complement cascade and Porphyromonas gingivalis in Alzheimer's disease? *J Oral Microbiol.* (2020) 12:1676486. doi: 10.1080/20002297.2019.1676486
40. Dominy SS, Lynch C, Ermini F, Benedyk M, Marczyk A, Konradi A, et al. Porphyromonas gingivalis in Alzheimer's disease brains: evidence for disease causation and treatment with small-molecule inhibitors. *Sci Adv.* (2019) 5:eaau3333. doi: 10.1126/sciadv.aau3333
41. Fitzpatrick RE, Wijeyewickrema LC, Pike RN. The gingipains: scissors and glue of the periodontal pathogen, Porphyromonas gingivalis. *Future Microbiol.* (2009) 4:471–87. doi: 10.2217/fmb.09.18
42. Singhrao SK, Olsen I. Assessing the role of Porphyromonas gingivalis in periodontitis to determine a causative relationship with Alzheimer's disease. *J Oral Microbiol.* (2019) 11:1563405. doi: 10.1080/20002297.2018.1563405
43. Zhang J, Yu C, Zhang X, Chen H, Dong J, Lu W, et al. Porphyromonas gingivalis lipopolysaccharide induces cognitive dysfunction, mediated by neuronal inflammation via activation of the TLR4 signaling pathway in C57BL/6 mice. *J Neuroinflammation.* (2018) 15:37. doi: 10.1186/s12974-017-1052-x
44. Nakayama M, Inoue T, Naito M, Nakayama K, Ohara N. Attenuation of the phosphatidylinositol 3-kinase/Akt signaling pathway by Porphyromonas gingivalis gingipains RgpA, RgpB, and Kgp. *J Biol Chem.* (2015) 290:5190–202. doi: 10.1074/jbc.M114.591610
45. Guo Y, Nguyen KA, Potempa J. Dichotomy of gingipains action as virulence factors: from cleaving substrates with the precision of a surgeon's knife to a meat chopper-like brutal degradation of proteins. *Periodontol.* (2000) 54:15–44. doi: 10.1111/j.1600-0757.2010.00377.x
46. Travis J, Pike R, Imamura T, Potempa J. Porphyromonas gingivalis proteinases as virulence factors in the development of periodontitis. *J Periodontal Res.* (1997) 32:120–5. doi: 10.1111/j.1600-0765.1997.tb01392.x
47. O' Brien-Simpson NM, Veith PD, Dashper SG, Reynolds EC. Porphyromonas gingivalis gingipains: the molecular teeth of a microbial vampire. *Curr Protein Pept Sci.* (2003) 4:409–26. doi: 10.2174/1389203033487009
48. Ally N, Whisstock JC, Sieprawska-Lupa M, Potempa J, Le Bonniec BF, Travis J, et al. Characterization of the specificity of arginine-specific gingipains from Porphyromonas gingivalis reveals active site differences between different forms of the enzymes. *Biochemistry.* (2003) 42:11693–700. doi: 10.1021/bi0349726
49. Pretorius E. Platelets as potent signaling entities in Type 2 diabetes mellitus. *Trends Endocrinol Metab.* (2019) 30:532–45. doi: 10.1016/j.tem.2019.05.003
50. Pretorius E, Bester J, Vermeulen N, Lipinski B. Oxidation inhibits iron-induced blood coagulation. *Curr Drug Targets.* (2013) 14:13–9. doi: 10.2174/138945013804806541
51. Soma P, Swanepoel AC, du Plooy JN, Mqoco T, Pretorius E. Flow cytometric analysis of platelets type 2 diabetes mellitus reveals 'angry' platelets. *Cardiovasc Diabetol.* (2016) 15:52. doi: 10.1186/s12933-016-0373-x
52. Pretorius E, Page MJ, Hendricks L, Nkosi NB, Benson SR, Kell DB. Both lipopolysaccharide and lipoteichoic acids potently induce anomalous fibrin amyloid formation: assessment with novel Amytracker stains. *J R Soc Interface.* (2018) 15:20170941. doi: 10.1098/rsif.2017.0941
53. Senini V, Amara U, Paul M, Kim H. Porphyromonas gingivalis lipopolysaccharide activates platelet Cdc42 and promotes platelet spreading and thrombosis. *J Periodontol.* (2019) 90:1336–45. doi: 10.1002/JPER.18-0596
54. Adams B, Nunes JM, Page MJ, Roberts T, Carr J, Nell TA, et al. Parkinson's disease: a systemic inflammatory disease accompanied by bacterial inflammagens. *Front Aging Neurosci.* (2019) 11:210. doi: 10.3389/fnagi.2019.00210
55. Joshi C, Bapat R, Anderson W, Dawson D, Hijazi K, Cherukara G. Detection of periodontal microorganisms in coronary atheromatous plaque specimens of myocardial infarction patients: a systematic review and meta-analysis. *Trends Cardiovasc Med.* (2019). doi: 10.1016/j.tcm.2019.12.005. [Epub ahead of print].
56. Jain S, Coats SR, Chang AM, Darveau RP. A novel class of lipoprotein lipase-sensitive molecules mediates toll-like receptor 2 activation by Porphyromonas gingivalis. *Infect Immun.* (2013) 81:1277–86. doi: 10.1128/IAI.01036-12
57. Pretorius E, Swanepoel AC, DeVilliers S, Bester J. Blood clot parameters: Thromboelastography and scanning electron microscopy in research and clinical practice. *Thromb Res.* (2017) 154:59–63. doi: 10.1016/j.thromres.2017.04.005
58. Argatov I, Iantchenko A, Kocherbitov V. How to define the storage and loss moduli for a rheologically nonlinear material? *Continuum Mech Thermodyn.* (2017) 29:1375–87. doi: 10.1007/s00161-017-0584-8
59. Ewoldt RH, Hosoi AE, McKinley GH. New measures for characterizing nonlinear viscoelasticity in large amplitude oscillatory shear. *J Rheometry.* (2008) 52:1427. doi: 10.1122/1.2970095
60. Hyun K, Wilhelm M, Klein CO, Cho SK, Nam JG, Ahn KH, et al. A review of nonlinear oscillatory shear tests: analysis and application of large amplitude oscillatory shear (LAOS). *Prog Polym Sci.* (2011) 36:1697–753. doi: 10.1016/j.progpolymsci.2011.02.002
61. Page MJ, Thomson GJA, Nunes JM, Engelbrecht AM, Nell TA, de Villiers WJS, et al. Serum amyloid A binds to fibrin(ogen), promoting fibrin amyloid formation. *Sci Rep.* (2019) 9:3102. doi: 10.1038/s41598-019-39056-x
62. Pike R, McGraw W, Potempa J, Travis J. Lysine- and arginine-specific proteinases from Porphyromonas gingivalis. Isolation, characterization, and evidence for the existence of complexes with hemagglutinins. *J Biol Chem.* (1994) 269:406–11.
63. Ben Amar M. *New Trends in the Physics and Mechanics of Biological Systems.* Oxford Scholarship Online (2001). doi: 10.1093/acprof:oso/9780199605835.001.0001
64. Tam V, O'Brien-Simpson NM, Chen YY, Sanderson CJ, Kinnear B, Reynolds EC. The RgpA-Kgp proteinase-adhesin complexes of Porphyromonas gingivalis Inactivate the Th2 cytokines interleukin-4 and interleukin-5. *Infect Immun.* (2009) 77:1451–8. doi: 10.1128/IAI.01377-08
65. O'Brien-Simpson NM, Paolini RA, Hoffmann B, Slakeski N, Dashper SG, Reynolds EC. Role of RgpA, RgpB, and Kgp proteinases in virulence of Porphyromonas gingivalis W50 in a murine lesion model. *Infect Immun.* (2001) 69:7527–34. doi: 10.1128/IAI.69.12.7527-7534.2001

Conflict of Interest: OL is employed by the company, Horiba. She analysed the sample using Raman technology.

The remaining 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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Oral Microbiome and Gingival Tissue Apoptosis and Autophagy Transcriptomics

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Objective: This study focused on documenting characteristics of the gingival transcriptome during various stages of periodontitis targeting genes associated with apoptotic and autophagic pathways and changes that specifically associate with features of the oral microbiome.

Methods: *Macaca mulatta* ($n = 18$; 12–23 years) were examined at baseline and 0.5, 1, and 3 months of disease progression, as well as 5 months with clinical disease resolution. 16S sequencing and microarray analyses examined changes in the microbiome and gingival transcriptome, respectively, at each time point from every animal.

Results: Specific patterns of apoptotic and autophagic genes were identified related to the initiation and progression of disease. The analysis also provided insights on the principal bacteria within the complex microbiome whose abundance was significantly correlated with differences in apoptotic and autophagic gene expression. Bacteria were identified that formed associated complexes with similar effects on the host gene expression profiles. A complex of *Leptotrichia*_unclassifed, *Capnocytophaga*_unclassified, *Prevotella* sp. 317, and *Veillonellaceae*_[G-1] sp. 155 were significantly negatively correlated with both apoptosis and autophagy. Whereas, *Veillonellaceae*_[G-1], *Porphyromonadaceae*, and *F. alocis* 539 were significantly positively correlated with both pathways, albeit this relationship was primarily associated with pro-apoptotic genes.

Conclusions: The findings provide evidence for specific bacteria/bacterial complexes within the oral microbiome that appear to have a more substantive effect on regulating apoptotic and autophagic pathways in the gingival tissues with periodontitis.

Keywords: non-human primates, apoptosis, autophagy, microbiome, periodontitis

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Apoptosis and autophagy are described as important regulators of cellular functions, particularly related to nutritional stress that could be induced by infection and inflammation. As such, recent results support an important role for each of these cell death or survival pathways in regulating inflammatory and immune responses. Periodontitis represents a complex chronic microbial challenge by a dysbiotic microbiome that results in a persistent inflammatory response and a tissue

destructive immunoinflammatory lesion. Although there are clear clinical symptoms of this disease including soft and hard tissue destruction of the periodontium, the underlying biology of the host-bacterial interactions that lead to initiation, progression, and resolution of the disease remain less clear. We have used a non-human primate model of ligature-induced experimental periodontal disease to try to model these host-bacterial interactions.

INTRODUCTION

Mucosal surfaces of the body, including the oral cavity, are continually interacting with a complex microbiome (1). This challenge results in activation of an array of immune response pathways, and cells and biomolecules that maintain homeostasis. Moreover, the oral cavity presents a model of mucosal host-bacterial interactions whereby specific microorganisms colonize various niches (2) creating complex biofilms that change with local environmental cues and respond to disease processes of the periodontium (3–6).

Chronic periodontitis reflects an active breakdown of connective tissue and resorption of alveolar bone, with localized lesions attributed to a dysregulated host inflammatory and immune responses to dysbiotic microbial biofilms (7–12). Microbiome changes with disease have been shown to include alterations in specific members of the subgingival microbiome at disease sites (e.g., *Porphyromonas gingivalis*) with the capacity to alter the biologic activities of the overall microbiome, as well as regulating host responses that would contribute to the tissue breakdown as a hallmark of periodontitis (13, 14).

The gingival mucosal tissues are enriched in cellular turnover with many cells undergoing programmed cell death (*i.e.,* apoptosis) (15). Apoptotic processes appear important for natural maintenance of an intact epithelial barrier that contributes anti-microbial resistance (16) and appears to be an important mechanism that regulates the immunoinflammatory response to microbial challenge (15, 17, 18). Moreover, autophagy is a well-described mechanism for host cells to engulf microbes or damaged cell material into autophagosomes for eventual degradation (19). Cells with defective autophagy pathways exhibit exaggerated inflammation and increased susceptibility to infections (20–23). Additionally, various microbial species appear to modulate autophagy as a virulence strategy to enable persistent survival inside host cells affecting both anti-microbial and anti-inflammatory responses (24, 25).

The characterization of both apoptosis and autophagy as having important roles in infection and inflammation is a relatively recent concept. The impact of these cellular functions on anti-microbial and anti-inflammatory properties suggests that alterations could contribute to the pathogenesis of periodontitis. Existing data indicates that specific bacteria within the oral ecology produce components that inhibit apoptotic pathways (26–28) and can modulate autophagic responses, including *P. gingivalis* (26, 29, 30). These functions have been described to enable evasion of responses and persistent infection of the oral epithelium, and even enhance survival inside endothelial cells (31). Knowledge of the role of apoptosis and autophagy in periodontitis remains rather limited and thus studies of these pathways in mucosal tissues related to gingival health and periodontitis are clearly needed.

We have described gene expression profiles in gingival tissues from young to aged non-human primates, *Macaca mulatta*, and demonstrated altered patterns of apoptotic (32, 33) and autophagic (34) pathway genes affected by aging and periodontitis. Imbalances in these processes would be consistent with the capacity to respond to the microbial challenge, as well as a disruption related to the disease process (35–37). This study focused on the analysis of the expression of targeted gene sets related to the pathways of apoptosis and various phases of autophagy pathways using the non-human primate model of progressing periodontitis. The gingival transcriptome expression was specifically integrated into a model exploring the relationship between characteristics of the oral microbiome matched to gingival sites in health and progressing disease.

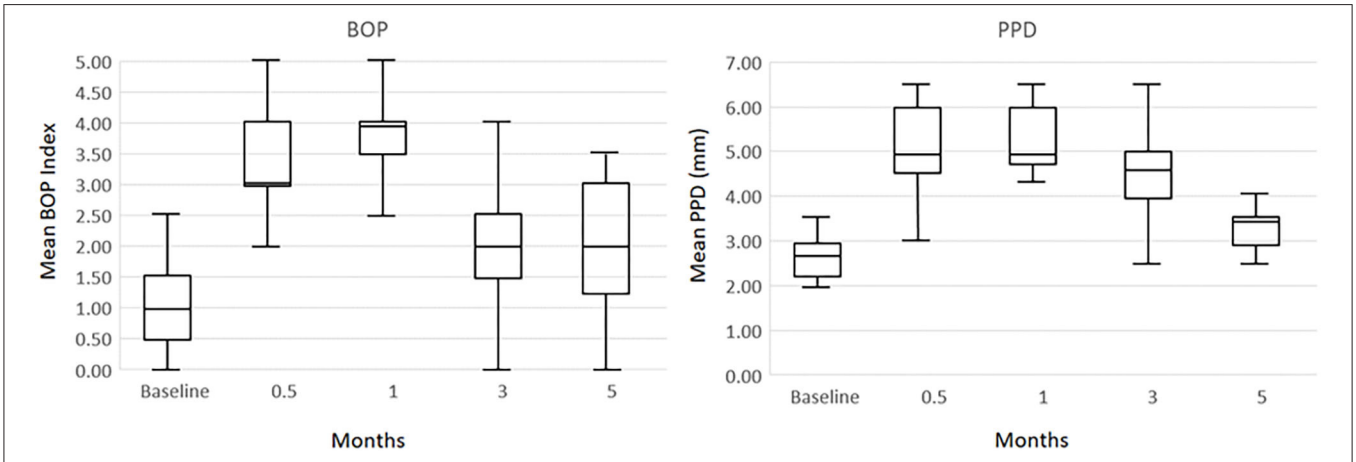

FIGURE 1 | Boxplot of clinical measures of ligated teeth in the 18 animals at each time point for bleeding on probing (BOP) index and probing pocket depth (PPD).

MATERIALS AND METHODS

Animals and Diet

Rhesus monkeys (*Macaca mulatta*) ($n = 18$; 12–23 years of age) housed at the Caribbean Primate Research Center at Sabana Seca, Puerto Rico were examined for periodontal health or naturally-occurring periodontitis (32, 33, 38). The non-human primates were fed a 20% protein, 5% fat, and 10% fiber commercial monkey diet (diet 8773, Teklad NIB primate diet modified: Harlan Teklad, Madison, WI). The diet was supplemented with

TABLE 1 | Listing of host genes examined for apoptosis and autophagy processes in the gingival tissues.

Gene ID	Gene name	Fxn	Gene ID	Gene name	Fxn
Apoptosis			**Autophagy**		
AKT3	AKT Serine/Threonine Kinase 3	A	EIF4G1	Eukaryotic Translation Initiation Factor 4 Gamma 1	MTOR
BIRC3	Baculoviral IAP Repeat Containing 3	A	MAPKSP1/ LAMTOR3	Late Endosomal/Lysosomal Adaptor, MAPK and, MTOR Activator 3	MTOR
CD2	CD2 Molecule	A	ATG101	Autophagy Related 101	ULK
CFLAR	CASP8 and FADD Like Apoptosis Regulator	A	DRAM1	DNA Damage Regulated Autophagy Modulator 1	ULK
CSF2RB	Colony Stimulating Factor 2 Receptor Subunit Beta	A	DRAM2	DNA Damage Regulated Autophagy Modulator 2	ULK
NOL3	Nucleolar Protein 3	A	SNX4	Sorting Nexin 4	ULK
PIK3CD	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta	A	ATG14/Barkor	Autophagy Related 14	PI3K
PIK3CG	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Gamma	A	DAPK1	Death Associated Protein Kinase 1	PI3K
PRKACB	Protein Kinase CAMP-Activated Catalytic Subunit Beta	A	IGF1	Insulin Like Growth Factor 1	PI3K
APAF1	Apoptotic Peptidase Activating Factor 1	P	PIK3CG	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Gamma	PI3K
ATM	ATM Serine/Threonine Kinase	P	RB1	RB Transcriptional Corepressor 1	PI3K
BID	BH3 Interacting Domain Death Agonist	P	SHIP2/INPPL1	Inositol Polyphosphate Phosphatase Like 1	PI3K
CASP1	Caspase 1	P	ATG3	Autophagy Related 3	ATG12
CASP3	Caspase 3	P	ATG4C	Autophagy Related 4C	ATG12
CASP7	Caspase 7	P	ATG4D	Autophagy Related 4D	ATG12
CASP8	Caspase 8	P	ATG5	Autophagy Related 5	ATG12
CASP10	Caspase 10	P	ATG7	Autophagy Related 7	ATG12
DAPK1	Death Associated Protein Kinase 1	P	ATG16L2	Autophagy Related Like 2	ATG12
ENDOD1	Endonuclease Domain Containing 1	P	CALCOCO2	Calcium Binding and Coiled-Coil Domain 2	ATG12
FAS	Fas Cell Surface Death Receptor	P	CXCR4	C-X-C Motif Chemokine Receptor 4	ATG12
IL1A	Interleukin-1 Alpha	P	EIF2AK3/PERK	Eukaryotic Translation Initiation Factor 2 Alpha Kinase 3	ATG12
IL1B	Interleukin-1 Beta	P	EIF2AK4/GCN2	Eukaryotic Translation Initiation Factor 2 Alpha Kinase 4	ATG12
IL1R1	Interleukin 1 Receptor Type 1	P	GABARAPL2/ ATG8c	GABA Type A Receptor Associated Protein Like 2	ATG12
IL1RAP	Interleukin 1 Receptor Accessory Protein	P	PLIN2	Perilipin 2	ATG12
IRAK3	Interleukin 1 Receptor Associated Kinase 3	P	BAD	BCL2 Associated Agonist Of Cell Death	LF/VD
PRKAR2B	Protein Kinase CAMP-Dependent Type II Regulatory Subunit Beta	P	BAK1	BCL2 Antagonist/Killer 1	LF/VD
TNFRS11B	TNF Receptor Superfamily Member 11b	P	CASP8	Caspase 8	LF/VD
TRAF3	TNF Receptor Associated Factor 3	P	CTSL2/CTSV	Cathepsin V	LF/VD
			EPAS1	Endothelial PAS Domain Protein 1	LF/VD
			FAS	Fas Cell Surface Death Receptor	LF/VD
			LAMP2	Lysosomal Associated Membrane Protein 2	LF/VD
			PRKCQ	Protein Kinase C Theta	LF/VD
			VAMP8	Vesicle Associated Membrane Protein 8	LF/VD

Functions denote: anti-apoptosis (A), pro-apoptosis (P), mTORC1 complex (mTOR), ULK complex (ULK), PI3K complex (PI3K), ATG12 interactions (ATG12), and lysosome fusion/vesicle degradation (LF/VD).

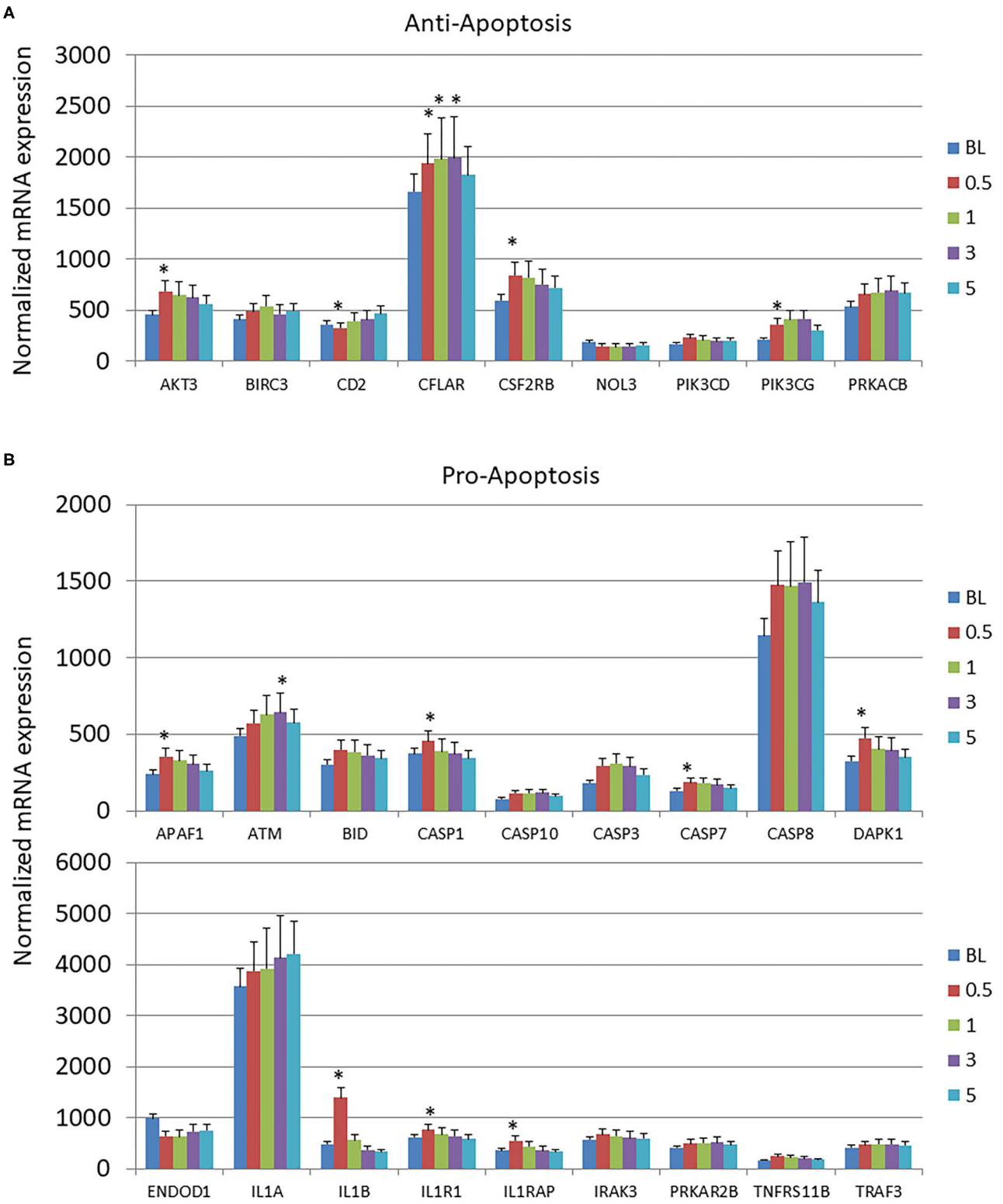

FIGURE 2 | Expression levels of **(A)** anti-apoptotic and **(B)** pro-apoptotic genes in gingival samples at baseline (BL) and 0.5, 1, and 3 months following ligation. Five month samples represent 60 days following removal of the ligatures with resolution of the clinical features of the disease. The bars denote the means of 18 animal samples and the vertical bracket signifies one SD. The asterisk (*) denotes significantly different from baseline levels at $p < 0.05$.

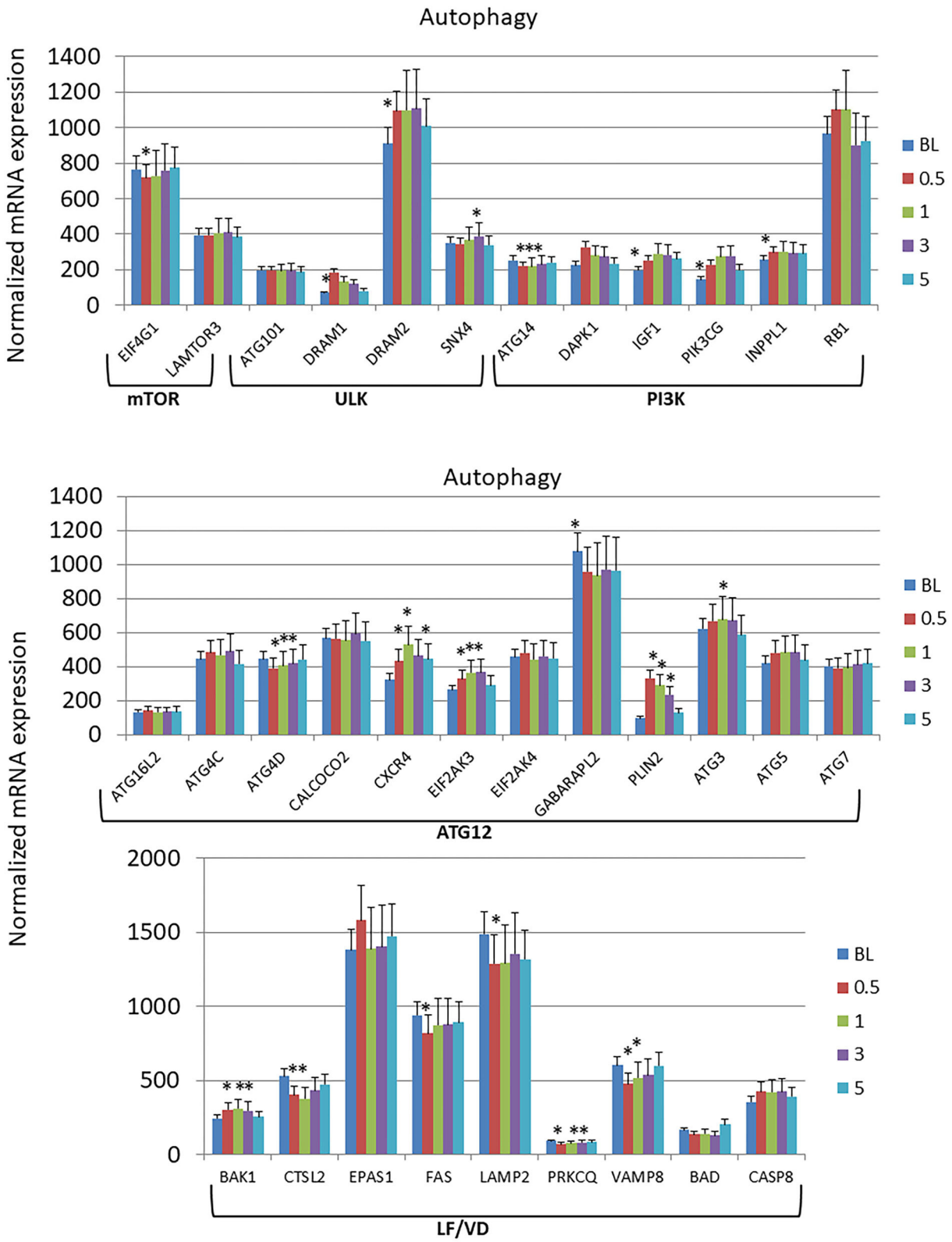

FIGURE 3 | Expression levels of autophagy genes in gingival samples at baseline (BL) and 0.5, 1, and 3 months following ligation. Five month samples represent 60 days following removal of the ligatures with resolution of the clinical features of the disease. The genes are grouped into various categories representing steps in the autophagy pathway. The bars denote the means of 18 animal samples and the vertical bracket signifies one SD. The asterisk (*) denotes significantly different from baseline levels or baseline levels being different from all other time points at $p < 0.05$.

fruits and vegetables, and water was provided *ad libitum* in an enclosed corral setting.

As we have reported previously the protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico and a ligature disease model was utilized (39). The clinical examination included probing pocket depth (PPD) and bleeding on probing (BOP; 0–5 scale) (40). Periodontal health was defined by mean Pocket Depth (PD) $\leq$ 3.0 mm and mean Bleeding on Probing (BOP) $\leq$ 1 (0–5 scale) in a full mouth examination excluding 3rd molars and canines (39). Ligature-induced periodontal disease was initiated as we have previously reported and clinical changes were compared to baseline measures of all maxillary and mandibular premolars and 1st and 2nd molars that were then ligated. The ligature-induced periodontitis model was implemented as we have described previously, by tying 3-0 silk sutures around the necks of maxillary and mandibular premolar and 1st and 2nd molar teeth in each animal (41, 42). As noted previously, removal of ligatures leads to a decrease in inflammation and BOP to normal levels and stabilization of any pocket probing depths in the non-human primates (42). Gingiva tissue biopsies and subgingival plaque samples taken at 0.5, 1, and 3 months (Initiation/Progression), and 2 months after removal of ligatures and local factors (Resolution). Determination of periodontal disease at the sampled site was documented by assessment of the presence of BOP and probing pocket depth of > 4 mm, as we have described previously (33). **Figure 1** presents the clinical findings for both bleeding on probing and probing pocket depth for the 18 animals. As shown previously, these clinical measures of periodontal inflammation and tissue changes occur as early as 0.5 months post-ligature (Baseline), and progress through the 3 months of disease progression.

Microbiome Analysis

Subgingival bacterial samples were obtained from the 18 animals by a curette and analyzed using a MiSeq instrument (43, 44) for the total composition of the microbiome from each sample (45, 46). Sequences were clustered into phylotypes based on their sequence similarity and these binned phylotypes were assigned to their respective taxonomic classification using the Human Oral Microbiome Database (HOMD V13) (http://www.homd.org/index.php?name=seqDownload&file&type$=$R) as we have described previously (43). Raw data were deposited at the NIH NCBI (BioProject ID PRJNA516659). Statistical differences of bacterial OTUs were determined with a *t-test* ($p < 0.05$). Correlations of OTUs within the oral microbiome were determined using a Pearson correlation coefficient analysis ($p < 0.05$). Correlations between the microbiome components and the gingival gene expression were determined only for matching samples derived from the same tooth in each of the animals. Matching samples with sufficient microbiome signals were compared for 58 samples obtained throughout the ligature model.

Gingival Tissue Sample Collection and mRNA Analysis

Gingival tissue samples of healthy and disease sites were surgically collected and total RNA extracted for microarray analysis via hybridization to the GeneChip® Rhesus Gene 1.0 ST

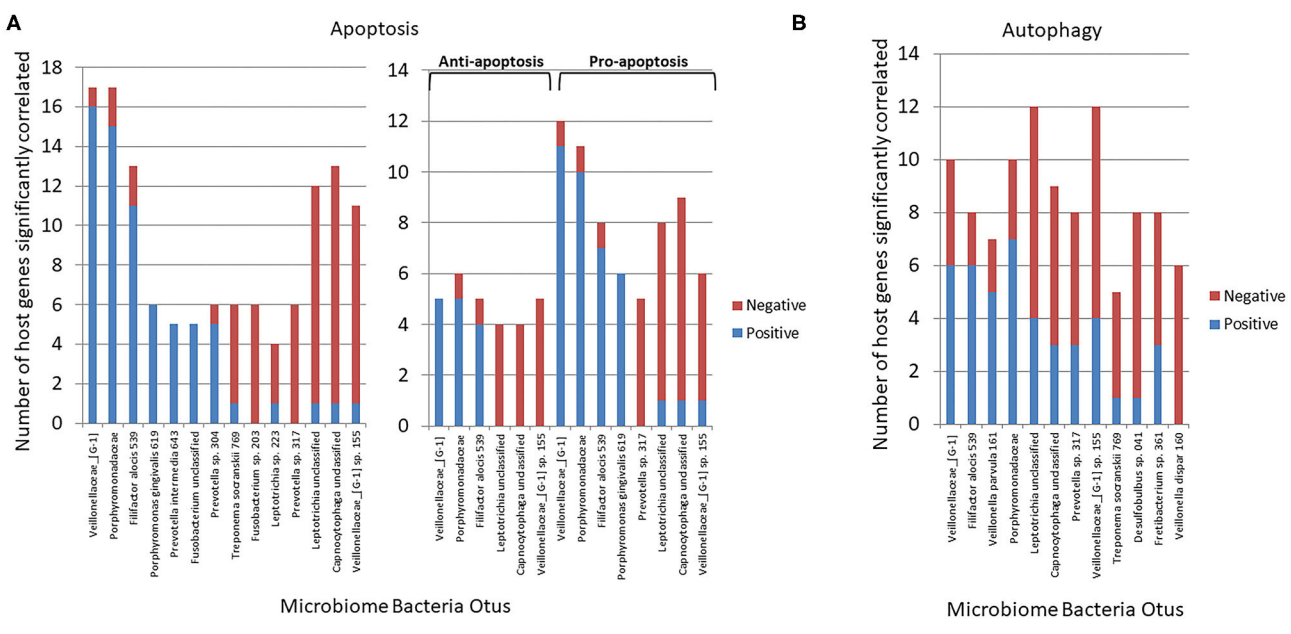

FIGURE 4 | Correlations ($p < 0.05$) between individual bacterial abundance within the microbiome to gene expression levels from the same sites. **(A)** Frequency of correlations for all the apoptosis genes for each bacterial OTU and stratified based upon significant correlations specifically with anti-apoptosis or pro-apoptosis genes. **(B)** Frequency of correlations for all autophagy genes for each bacterial OTU.

Array (Affymetrix, Santa Clara, CA, USA) similar to methods we have described previously (32, 33, 47–49).

Data Analysis

Pro- and anti-apoptosis pathway genes ($n = 27$) and 33 genes involved in the autophagy pathway (**Table 1**) that we had previously identified in non-human primates as being affected by age or periodontitis were targeted. The expression intensities across the samples were estimated using the Robust Multi-array Average (RMA) algorithm with probe-level quintile normalization, as implemented in the Partek Genomics Suite software version 6.6 (Partek, St. Louis, MO). The different groups were initially compared using one way ANOVA. For genes that had significant mean differences, two sample *t*-tests were used to investigate differences. Statistical significance was considered by a $p \leq 0.05$. Correlation analyses between the microbiome Otus and the gingival gene expression were performed using a Spearman rank-order correlation analysis. The data has been uploaded into the ArrayExpress data base (www.ebi.ac.uk) under accession number: E-MTAB-1977.

RESULTS

Longitudinal Changes in Apoptosis Gene Expression Profiles

Few changes from baseline healthy tissues were observed in the anti-apoptosis genes across the disease process or with resolution (**Figure 2A**). In contrast, **Figure 2B** summarizes that 8/18 of the pro-apoptosis genes were significantly altered through the disease process. The majority of these changes were increases occurring at initiation of disease.

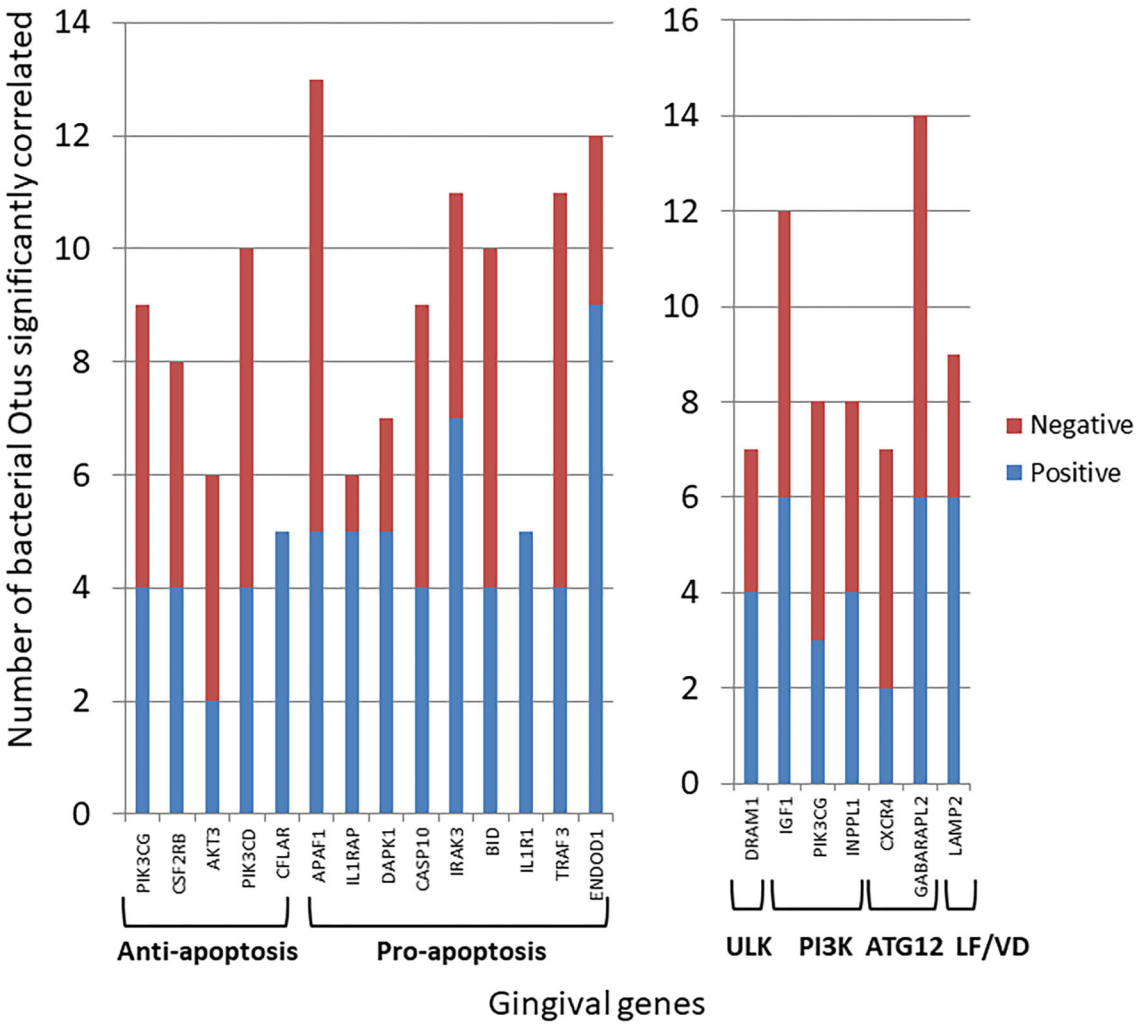

FIGURE 5 | Individual gene expression levels correlated with bacterial abundance levels in the microbiome samples. The stacked bars depict the frequency of microbial correlations of a particular apoptotic or autophagy gene separated into significant ($p < 0.05$) positive or negative correlations. Each gene is organized into apoptosis processes or steps in the autophagy pathway. The genes presented had > 7 total bacterial correlations except for CFLAR and IL1R1 that showed a unique pattern of only positive correlations.

Longitudinal Changes in Autophagy Gene Expression Profiles

The autophagy gene expression profiles were organized into 6 phases of the process including initial interactions through the Mechanistic Target Of Rapamycin Kinase (mTOR) and Unc-51 Like Autophagy Activating Kinase 1 (ULK) complexes, signaling activities via genes in the PI3K (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase) complex, expansion of the phagophore via Autophagy Related 12 (ATG12)-related genes, and finally linked to lysosome formation and vesicle degradation (LF/VD) processes (**Figure 3**). Generally in the early stages of the process (mTOR, ULK, PI3K), gene expression alterations were increased from baseline health to initiation and progression of disease. A larger number of gene expression level changes were identified in the ATG12 portion of the autophagy pathway with most demonstrating elevated levels compared to health (baseline). Only ATG4D and GABA Type A Receptor Associated Protein Like 2 (GABARAPL2; ATG8C) levels were decreased with disease. Finally, LF/VD gene expression appeared to be decreased during disease.

Microbiome Members Associated With Altered Apoptosis and Autophagy Gene Expression

Figure 4A summarizes the correlation patterns of the 22 apoptosis genes with specific bacteria among the 58 OTUs that account for 88% of the total microbial reads across the adult samples (43). Specific OTUs were significantly correlated with a high number of host genes, and displayed as ones with a majority of positive or negative correlations (**Supplementary Table 1**). The data also show that most of these OTUs had a predilection for either positive or negative correlations with the array of apoptotic genes. Also of note was that in all comparisons the correlations of these bacteria predominated with expression of pro-apoptotic genes either positively (*Veillonellaceae*_[G1], *Porphyromonadaceae, F. alocis* 539) or negatively (*Prevotella* sp. 317, *Leptotrichia* unclassified, *Capnocytophaga* unclassified). **Figure 4B** presents the relationship of the OTUs to the autophagy genes. While the bacteria (e.g., OTUs) generally showed both positive and negative correlations to genes within the complex of the autophagy pathway, as with apoptosis the individual OTUs were skewed toward a higher frequency of positive or negative correlations (**Supplementary Table 2**). Of interest was a considerable overlap in the specific OTUs of autophagy and apoptosis gene correlations, with ∼2/3 of the OTUs overlapping.

Transposing these data provided insight into the primary genes within the apoptosis or autophagy pathways demonstrating preferential correlations with the microbiome members across all the samples (**Figure 5**). These included both anti- and pro-apoptotic genes with most showing a similar number of bacteria exhibiting positive or negative correlations. A more limited number of autophagy genes across the pathway phases showed significant correlations with a larger number of individual bacteria, and as with apoptosis, there was a similar distribution of positively, and negatively correlated bacteria.

TABLE 2 | Complexes of bacteria with related significant correlations to apoptosis genes.

ID	Bacterial complex	Anti	Pro
AP-I	Veillonellaceae_[G-1] Porphyromonadaceae Filifactor alocis 539 Porphyromonas gingivalis 619 Prevotella intermedia 643 Fusobacterium unclassified *Prevotella* sp. 304	PI3KCG CSF2RB AKT3 PIK3CD CFLAR NOL3	IL1B TNFRS118 APAFI ILIRAP CASP7 DAPKI IRAK3 BID IL1R1 TRAF3 ENDOD1
AP-2	*Desulfobulbus* sp. 041 Treponema socranskii 769 *Fusobacterium* sp. 203 *Leptotrichia* sp. 223 Selenomonas unclassified *Prevotella* sp. 317 Leptotrichia uncla ssified Capnocytophaga unclassified *Veillonellaceae_[G-I]* sp. 155	PI3KCG CSF2RB AKT3 PIK3CD	CASP3 APAF1 CASP10 IRAK3 BID TRAF3 ENDOD1
AP-3	Porphyromonadaceae Prevotella intermedia 643 *Prevotella* sp. 313 Veillonella dispar 160		FAS

The genes in Green are positively correlated and in Red are negatively correlated with the bacterial complexes.

Bacterial Complexes Related to Altered Apoptosis and Autophagy Gene Expression Profiles

Based upon the observation that there were multiple bacterial-host gene correlations for both apoptosis and autophagy, we explored the potential that complexes of certain bacteria demonstrated similar patterns of interaction with host responses across the disease model. **Table 2** shows 3 bacterial complexes (AP-1 to AP-3) that were related in their frequency of correlations with a specific portfolio of anti- or pro-apoptotic genes. As noted, the same complex could show both significantly positive and negative correlations to these panels of different host genes. **Table 3** provides a similar summary where 5 complexes (AU-1 to AU-5) that were identified to show significant correlations with multiple host genes across the autophagy pathway. Interestingly, there was not only considerable overlap in the complex members identified related to apoptosis and autophagy gene expression, but there were similarities in the specific complex groupings correlated with the gene expression levels. This was demonstrated by representatives in the AP-1/AU-2 complexes, AP-2/AU-1 complexes, and AP-3/AU-5 complexes.

Bacterial Complexes Affecting Apoptosis and Autophagy Pathways

Figure 6 presents a KEGG pathway schematic for apoptosis. The three bacterial complexes are included and genes correlated (positively or negatively) are highlighted in the pathway. As

TABLE 3 | Complexes of bacteria with related significant correlations to autophagy genes.

ID	Bacterial complex	mTOR	ULK	PI3K	ATG9	ATG12	LF/VD
AU-I	Leptotrichia unclassified Capnocytophaga unclassified *Prevotella* sp. 317 *Veillonellaceae_[G-1]* sp. 155 Treponema socranskii 769 *Desulfobulbus* sp. 041	EIF4G1	DRAM2	IGF1 PIK3CG DAPK1 PIK3CG INPPL1		ATG4D CXCR4 EIF2AK3 GABARAPI2 PIIN2	LAMP2
AU-2	Veillonellaceae_[G-1] Fusobacterium unclassified Filifactor alocis 539		DRAM1				
AU-3	Prevotella fusca 782 *Prevotella* sp. 304 *SR1_[G-1]* sp. 345 Prevotella unclassified Pyramidobacter piscolens 357 Bacteria_unclassified		SNX4				
AU-4	Fretibacterium fastidiosum 363 Bacteroidetes_unclassified *Chloroflexi_[G-1]* sp. 439 *Fretibacterium* sp. 361		ATG101			GABARAPL2	
AU-5	Porphyromonadaceae Prevotella intermedia 643 *Prevotella* sp. 313 Veillonella dispar 160	EIF4G1	ATG101			EIF2AK4	EPAS1 FAS

The genes in Green are positively correlated and in Red are negatively correlated with the bacterial complexes.

can be seen, the complexes not only appear to impact multiple points in the pathway, but these complexes overlap with certain pathway points and generally affect the genes in a similar direction. In particular, these correlated bacterial complexes are generally associated with positive relaionships (i.e., higher bacteria/higher mRNA; lower bacteria/lower mRNA) including both upstream signaling of the cellular pathway, as well as alterations in downstream molecules required to completing apoptosis outcomes. **Figure 7** provides a similar schematic for the KEGG autophagy pathway. In this case the pathway steps that appear targeted by these bacterial complexes appear more limited with a predominance of bacterial complexes affecting the ULK initiation complex and three complexes negatively correlated with these genes two complexes negatively correlated with this initiation complex. Also, noted was that these bacterial complexes were also related to mTOR, ATG12, and LF/VD pathway steps with significant positive or negative correlations for gene regulation in the autophagy pathway.

DISCUSSION

Periodontal lesions develop from interactions of a dysbiotic oral microbiome and a juxtaposed dysregulated host response at sites of disease. There is clear evidence that the microbiome changes substantially from health to disease, with increases in overall burden, altered diversity, and emergence of various genera and species that are associated with and potentially causative of the host response dysregulation (50). However, the underlying mechanisms contributing to the breadth of host response changes including effects on resident cell functions, as well as alterations in infiltrating inflammatory and immune cells requires systems biology approaches and multi-omics analytics. These approaches are required to discern the earliest molecular stages of the disease process and transformations that occur for exacerbation or resolution of the lesion. As apoptosis and autophagy have been shown to be directly involved in the integrity of mucosal surfaces and regulation of localized inflammatory and immune responses, as well as likely contributing to the resolution of chronic inflammation, we sought to determine disease effects on these pathways and their relationship to microbiome conversion using a non-human primate model of ligature-induced periodontitis.

The results demonstrated specific alterations in the gingival transcriptome for both pro- and anti-apoptosis genes; however, the dominant changes with disease were increases in pro-apoptotic gene expression that generally occurred during disease initiation within 2 weeks of ligature placement and rapidly returned to baseline levels. Examination of changes in the autophagy-associated transcriptome, showed rather limited changes in the early events of the mTOR and ULK complexes. Interestingly, in the ULK complex both DRAM1 and DRAM2 were increased throughout disease, with both of these gene products important factors in regulating autophagy, and with increased combined levels also inducing apoptosis (51, 52). Some gene alterations in the PI3K complex were noted with increases during disease initiation. However, the most apparent alterations occurred in genes related to the ATG12 and lysosome fusion/vesicle degradation portions of the autophagy pathway. In this case, the ATG12 genes were generally increased with disease, while the LF/VD genes were

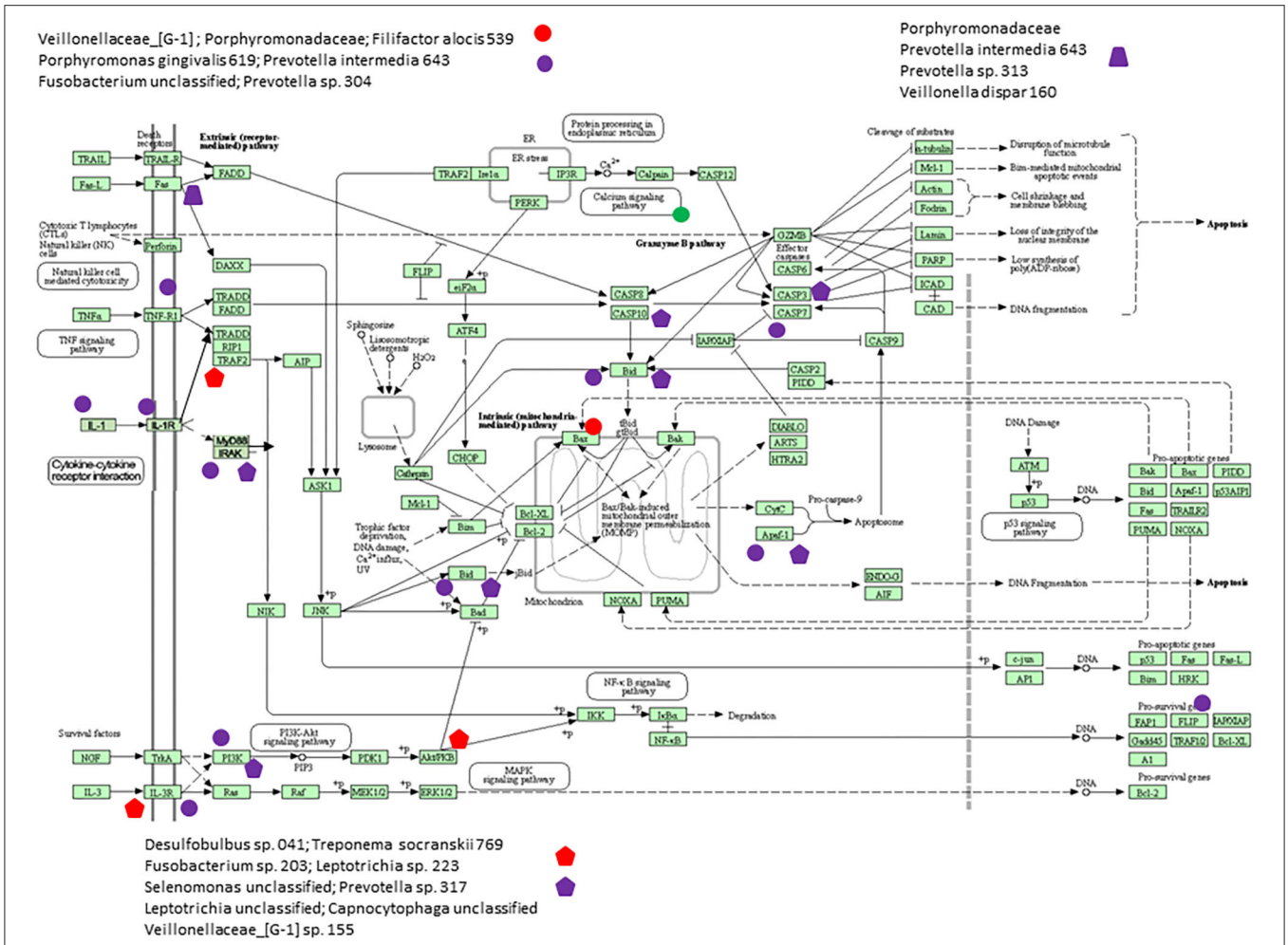

FIGURE 6 | KEGG pathway graphic for apoptosis. The bacterial complexes are identified with effects on apoptosis genes with the red colored symbols denoting a negative correlation and the purple colored symbols denoting a positive correlation with genes at various points in the pathway.

decreased with disease initiation and progression. Thus, based upon this profile, it appears that a complete functional autophagy pathway to aid in host protection, would be predicted to be less effective in supporting recovery from cellular stress during periodontitis.

Beyond the clear differences in the host gingival response, changes in the oral microbiome also occur during ligature-induced periodontitis, in many ways similar to the differences that have been reported with human disease (43, 53). This study identified unique features of the microbiome in disease that significantly correlated with these changes in the gingival transcriptome. Specifically among the entirety of the 396 OTUs that were identified across the non-human primate samples (43), we identified a rather focused set of microorganisms that related to the differential expression of both apoptosis and autophagy genes. The identified microbial complexes included some members of the oral microbiome historically considered as pathogens (e.g., *Porphyromonadaceae*, *P. gingivalis, F. alocis*), while some of the complexes that appear to robustly correlate with these host pathways, are generally considered members of the commensal microbiota. The data from complexes AP-1 and AP-2 are striking, as one complex predominantly showed positive correlations with both anti- and pro- apoptotic genes and the other skewed toward negative correlations. Thus, the AP-1 complex could be stimulating signals that block apoptosis, whereas the AP-2 complex could be enhancing it. As importantly, it would be considered peculiar to have host-bacterial interactions that activate both pro- and anti-apoptotic signals simultaneously with potential pathologic consequences. With pro-apoptotic pathways up-regulated the final outcome would be expected to be increases in cell death competing with survival pathways that are also activated. These counteracting functions could be occurring in the same cells, which could lead to a substantial metabolic cost and loss of normal functions, or may reflect members of these bacterial complexes demonstrating differential signaling of pro- or anti-apoptotic processes in different cell types within the periodontium. The details of these options do present a limitation of the study design. That is, these

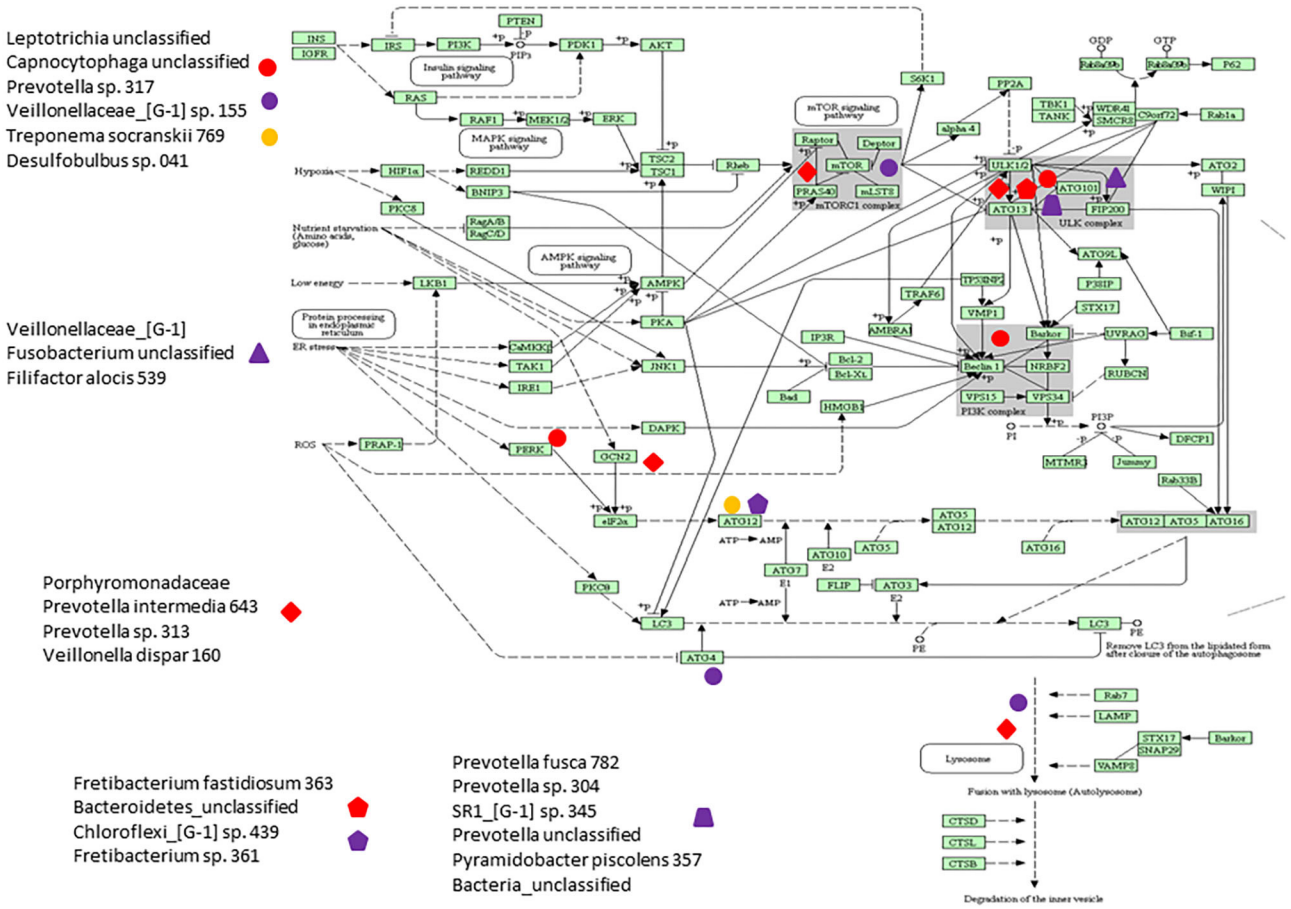

FIGURE 7 | KEGG pathway graphic for autophagy. The bacterial complexes are identified with a relationship to autophagy genes at various points in the pathway with the red colored symbols denoting a negative correlation, the purple colored symbols denoting a positive correlation, and and the yellow colored symbol denotes a bacterial complex that is correlated both positively and negatively with genes in the ATG12 portions of the autophagy pathway.

gene expression patterns represent a holistic evaluation of the complex tissues, including epithelial cells, fibroblasts/connective tissue, vascular, and inflammatory/immune cells within the mucosal tissues in health and disease. While an argument can be made regarding the usefulness of determining individual cell type gene expression profiles in the periodontium, there nevertheless is inherent value in fundamentally detailing the alterations in these pathways comparing healthy to disease tissues. The findings describe altered apoptosis and autophagy gene expression in these tissues that is specifically associated with the disease process and generally return to a pattern reflecting healthy tissues with disease resolution. Importantly, the study presents seminal data regarding the relationship between specific microbes or microbial complexes in the oral microbiome and altered gene expression for these pathways. Also of interest was that there were clear similarities of members of the complexes that appear to impact both apoptosis and autophagy and show comparable correlation directions in these relationships. In particular, a complex comprised of *Leptotrichia, Veillonella, Capnocytophaga, Prevotella, T. socranskii,* and *Desulfobulbus* appeared very active in the relationship with both pathways. Moreover, the correlation of this complex with the host responses appeared to reflect multiple factors within the pathways and effects on genes whose products would be expected to disrupt a needed balance to maintain homeostasis. This outcome could reflect fundamental capabilities of these bacteria and their components to stimulate host cellular changes in the gingival environment and be related most directly to increases in their relative abundance in the disease microbiome and potentially organized into synergistic interactions within the biofilms.

Alternatively, these findings might reveal features related to the concept of dysbiosis and disease. More specifically, these outcomes could result from changes in metabolism and transcriptome of these normal commensal bacteria toward expression of genes that would be more deleterious to host cells and tissues, whereby they would actually contribute to the disease environment and clinical outcomes (14, 50, 54). As

noted, simultaneous activation of pro-apoptotic pathways/genes and survival pathways might be considered pathologic at the individual cell level. This scenario could reflect an impaired cell death response that would contribute to persistent intracellular infection that can occur in periodontal tissues. Thus, some pathogens (e.g., *P. gingivalis, F. alocis*) and even commensals (e.g., *Veillonella*) could be involved in these impaired cell death responses by showing significant correlations with both pro- and anti-apoptotic gene expression. Additionally, the negative correlations with an increase in anti-apoptotic gene expression with decreased abundance of (OTUs) for specific bacteria, such as complex AP-2, could support a role for these particular bacterial species in inhibiting necessary apoptotic responses to maintain homeostasis. Further studies will be required to sort out these options, as well as determining the kinetics of changes occurring in the bacterial abundance that could contribute to a local environment that enhances tissue destructive events.

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 in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico.

AUTHOR CONTRIBUTIONS

JE and OG conceived of the studies, implemented the experiments, collected and analyzed the data, and prepared the manuscript. SK provided the analytics for the microbiome data and reviewing/revising the manuscript. EN provided an initial analysis of the host response data, an initial draft of the report, and reviewed the final manuscript. LO and JG provided the support for organizing the non-human primate studies and collection of samples and contributing to the methods section 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.2020.585414/full#supplementary-material

REFERENCES

1. Takayanagi H, Sato K, Takaoka A, Taniguchi T. Interplay between interferon and other cytokine systems in bone metabolism. *Immunol Rev.* (2005) 208:181–93. doi: 10.1111/j.0105-2896.2005.00337.x
2. Paster BJ, Olsen I, Aas JA, Dewhirst FE. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. *Periodontol.* (2006) 42:80–7. doi: 10.1111/j.1600-0757.2006.00174.x
3. Colombo AP, Boches SK, Cotton SL, Goodson JM, Kent R, Haffajee AD, et al. Comparisons of subgingival microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. *J Periodontol.* (2009) 80:1421–32. doi: 10.1902/jop.2009.090185
4. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol.* (2005) 43:5721–32. doi: 10.1128/JCM.43.11.5721-5732.2005
5. Kurzban GP, Chu L, Ebersole JL, Holt SC. Sulfhemoglobin formation in human erythrocytes by cystalysin, an L-cysteine desulfhydrase from Treponema denticola. *Oral Microbiol Immunol.* (1999) 14:153–64. doi: 10.1034/j.1399-302X.1999.140303.x
6. Periasamy S, Kolenbrander PE. Mutualistic biofilm communities develop with Porphyromonas gingivalis and initial, early, and late colonizers of enamel. *J Bacteriol.* (2009) 191:6804–11. doi: 10.1128/JB.01006-09
7. van Dyke TE, Winkelhoff AJv. Infection and inflammatory mechanisms. *J Periodontol.* (2013) 84:S1–7. doi: 10.1902/jop.2013.1340018
8. Ohlsson K, Olsson I, Tynelius-Bratthall G. Neutrophil leukocyte collagenase, elastase and serum protease inhibitors in human gingival crevicles. *Acta Odontol Scand.* (1974) 32:51–9. doi: 10.3109/00016357409002532
9. Chapple IL. Reactive oxygen species and antioxidants in inflammatory diseases. *J Clin Periodontol.* (1997) 24:287–96. doi: 10.1111/j.1600-051X.1997.tb00760.x
10. Chapple IL, Matthews JB. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol.* (2007) 43:160–232. doi: 10.1111/j.1600-0757.2006.00178.x
11. Halliwell B. Mechanisms involved in the generation of free radicals. *Pathol Biol.* (1996) 44:6–13.
12. Baltacioglu E, Akalin FA, Alver A, Deger O, Karabulut E. Protein carbonyl levels in serum and gingival crevicular fluid in patients with chronic periodontitis. *Arch Oral Biol.* (2008) 53:716–22. doi: 10.1016/j.archoralbio.2008.02.002
13. Solbiati J, Frias-Lopez J. Metatranscriptome of the oral microbiome in health and disease. *J Dent Res.* (2018) 97:492–500. doi: 10.1177/0022034518761644
14. Lamont RJ, Koo H, Hajishengallis G. The oral microbiota: dynamic communities and host interactions. *Nat Rev Microbiol.* (2018) 16:745–59. doi: 10.1038/s41579-018-0089-x
15. Duarte PM, Miranda TS, Lima JA, Dias Goncalves TE, Santos VR, Bastos MF, et al. Expression of immune-inflammatory markers in sites of chronic periodontitis in patients with type 2 diabetes. *J Periodontol.* (2012) 83:426–34. doi: 10.1902/jop.2011.110324
16. Cario E. Innate immune signalling at intestinal mucosal surfaces: a fine line between host protection and destruction. *Curr Opin Gastroenterol.* (2008) 24:725–32. doi: 10.1097/MOG.0b013e32830c4341
17. Duarte PM, Napimoga MH, Fagnani EC, Santos VR, Bastos MF, Ribeiro FV, et al. The expression of antioxidant enzymes in the gingivae of type 2 diabetics with chronic periodontitis. *Arch Oral Biol.* (2012) 57:161–8. doi: 10.1016/j.archoralbio.2011.08.007

18. Kim S, Elkon KB, Ma X. Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. *Immunity.* (2004) 21:643–53. doi: 10.1016/j.immuni.2004.09.009
19. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. *Cell.* (2010) 140:313–26. doi: 10.1016/j.cell.2010.01.028
20. Moscat J, Diaz-Meco MT. p62 at the crossroads of autophagy, apoptosis, and cancer. *Cell.* (2009) 137:1001–4. doi: 10.1016/j.cell.2009.05.023
21. Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol.* (2010) 12:213–23. doi: 10.1038/ncb2021
22. Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature.* (2008) 456:259–63. doi: 10.1038/nature07416
23. Mehrpour M, Botti J, Codogno P. Mechanisms and regulation of autophagy in mammalian cells. *Atlas Genet Cytogenet Oncol Haematol.* (2011) 16:165–82. doi: 10.4267/2042/46951
24. Levine B, Mizushima N, Virgin HW. Autophagy in immunity and inflammation. *Nature.* (2011) 469:323–35. doi: 10.1038/nature09782
25. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature.* (2008) 456:264–8. doi: 10.1038/nature07383
26. Taxman DJ, Swanson KV, Broglie PM, Wen H, Holley-Guthrie E, Huang MT, et al. Porphyromonas gingivalis mediates inflammasome repression in polymicrobial cultures through a novel mechanism involving reduced endocytosis. *J Biol Chem.* (2012) 287:32791–9. doi: 10.1074/jbc.M112.401737
27. Yao L, Jermanus C, Barbetta B, Choi C, Verbeke P, Ojcius DM, et al. Porphyromonas gingivalis infection sequesters pro-apoptotic Bad through Akt in primary gingival epithelial cells. *Mol Oral Microbiol.* (2010) 25:89–101. doi: 10.1111/j.2041-1014.2010.00569.x
28. Boisvert H, Duncan MJ. Translocation of Porphyromonas gingivalis gingipain adhesin peptide A44 to host mitochondria prevents apoptosis. *Infect Immun.* (2010) 78:3616–24. doi: 10.1128/IAI.00187-10
29. Bullon P, Cordero MD, Quiles JL, Ramirez-Tortosa Mdel C, Gonzalez-Alonso A, Alfonsi S, et al. Autophagy in periodontitis patients and gingival fibroblasts: unraveling the link between chronic diseases and inflammation. *BMC Med.* (2012) 10:122. doi: 10.1186/1741-7015-10-122
30. Tsai CC, Chen HS, Chen SL, Ho YP, Ho KY, Wu YM, et al. Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. *J Periodontal Res.* (2005) 40:378–84. doi: 10.1111/j.1600-0765.2005.00818.x
31. Rodrigues PH, Belanger M, Dunn W Jr, Progulske-Fox A. Porphyromonas gingivalis and the autophagic pathway: an innate immune interaction? *Front Biosci.* (2008) 13:178–87. doi: 10.2741/2668
32. Gonzalez OA, John Novak M, Kirakodu S, Stromberg AJ, Shen S, Orraca L, et al. Effects of aging on apoptosis gene expression in oral mucosal tissues. *Apoptosis.* (2013) 18:249–59. doi: 10.1007/s10495-013-0806-x
33. Gonzalez OA, Stromberg AJ, Huggins PM, Gonzalez-Martinez J, Novak MJ, Ebersole JL. Apoptotic genes are differentially expressed in aged gingival tissue. *J Dent Res.* (2011) 90:880–6. doi: 10.1177/0022034511403744
34. Ebersole JL, Kirakodu SS, Novak MJ, Dawson DI, Stromberg AJ, Orraca L, et al. Ginigval tissue autophagy pathway gene expression profiles in peirodontitis and aging. *J Periodontal Res*. (2020). doi: 10.1111/jre.12789. [Epub ahead of print].
35. Willershausen-Zonnchen B, Gleissner C. Periodontal disease in elderly patients. *Eur J Med Res.* (1998) 3:55–64.
36. Garcia RI, Krall EA, Vokonas PS. Periodontal disease and mortality from all causes in the VA dental longitudinal study. *Ann Periodontol.* (1998) 3:339–49. doi: 10.1902/annals.1998.3.1.339
37. Ship JA, Crow HC. Diseases of periodontal tissues in the elderly. Description, epidemiology, aetiology and drug therapy. *Drugs Aging.* (1994) 5:346–57. doi: 10.2165/00002512-199405050-00004
38. Ebersole JL, Steffen MJ, Reynolds MA, Branch-Mays GL, Dawson DR, Novak KF, et al. Differential gender effects of a reduced-calorie diet on systemic inflammatory and immune parameters in nonhuman primates. *J Periodontal Res.* (2008) 43:500–7. doi: 10.1111/j.1600-0765.2008.01051.x
39. Ebersole JL, Kirakodu S, Novak MJ, Stromberg AJ, Shen S, Orraca L, et al. Cytokine gene expression profiles during initiation, progression and resolution of periodontitis. *J Clin Periodontol.* (2014) 41:853–61. doi: 10.1111/jcpe.12286
40. Ebersole JL, Steffen MJ, Gonzalez-Martinez J, Novak MJ. Effects of age and oral disease on systemic inflammatory and immune parameters in nonhuman primates. *Clin Vaccine Immunol.* (2008) 15:1067–75. doi: 10.1128/CVI.00258-07
41. Reynolds M, Branch-Mays G, Dawson D III, Novak KF, Mattison J, Gunsolley J, et al. Effects of dietary calorie restriction on inflammatory periodontal disease. *Nutrition.* (2009) 95:88–97. doi: 10.1016/j.nut.2008.07.003
42. Branch-Mays GL, Dawson DR, Gunsolley JC, Reynolds MA, Ebersole JL, Novak KF, et al. The effects of a calorie-reduced diet on periodontal inflammation and disease in a non-human primate model. *J Periodontol.* (2008) 79:1184–91. doi: 10.1902/jop.2008.070629
43. Kirakodu S, Chen J, Gonzalez Martinez J, Gonzalez OA, Ebersole J. Microbiome profiles of ligature-induced periodontitis in nonhuman primates across the life Span. *Infect Immun.* (2019) 87:1–20. doi: 10.1128/IAI.00067-19
44. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol.* (2013) 79:5112–20. doi: 10.1128/AEM.01043-13
45. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol.* (2009) 75:7537–41. doi: 10.1128/AEM.01541-09
46. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics.* (2011) 27:2194–200. doi: 10.1093/bioinformatics/btr381
47. Gonzalez OA, Novak MJ, Kirakodu S, Stromberg A, Nagarajan R, Huang CB, et al. Differential gene expression profiles reflecting macrophage polarization in aging and periodontitis gingival tissues. *Immunol Invest.* (2015) 44:643–64. doi: 10.3109/08820139.2015.1070269
48. Gonzalez OA, Novak MJ, Kirakodu S, Orraca L, Chen KC, Stromberg A, et al. Comparative analysis of gingival tissue antigen presentation pathways in ageing and periodontitis. *J Clin Periodontol.* (2014) 41:327–39. doi: 10.1111/jcpe.12212
49. Meka A, Bakthavatchalu V, Sathishkumar S, Lopez MC, Verma RK, Wallet SM, et al. Porphyromonas gingivalis infection-induced tissue and bone transcriptional profiles. *Mol Oral Microbiol.* (2010) 25:61–74. doi: 10.1111/j.2041-1014.2009.00555.x
50. Curtis MA, Diaz PI, van Dyke TE. The role of the microbiota in periodontal disease. *Periodontol.* (2020) 83:14–25. doi: 10.1111/prd.12296
51. Tang N, Zhao H, Zhang H, Dong Y. Effect of autophagy gene DRAM on proliferation, cell cycle, apoptosis, and autophagy of osteoblast in osteoporosis rats. *J Cell Physiol.* (2019) 234:5023–32. doi: 10.1002/jcp.27304
52. Mrschtik M, Ryan KM. Another DRAM involved in autophagy and cell death. *Autophagy.* (2016) 12:603–5. doi: 10.1080/15548627.2015.1137412
53. Ebersole J, Kirakodu S, Chen J, Nagarajan R, Gonzalez OA. Oral microbiome and gingival transcriptome profiles of ligature-induced periodontitis. *J Dent Res.* (2020) 99:746–57. doi: 10.1177/0022034520906138
54. Kirst ME, Li EC, Alfant B, Chi YY, Walker C, Magnusson I, et al. Dysbiosis and alterations in predicted functions of the subgingival microbiome in chronic periodontitis. *Appl Environ Microbiol.* (2015) 81:783–93. doi: 10.1128/AEM.02712-14

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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REVIEW
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The Oral Microbiome and Cancer

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There is mounting evidence that members of the human microbiome are highly associated with a wide variety of cancer types. Among oral cancers, oral squamous cell carcinoma (OSCC) is the most prevalent and most commonly studied, and it is the most common malignancy of the head and neck worldwide. However, there is a void regarding the role that the oral microbiome may play in OSCC. Previous studies have not consistently found a characteristic oral microbiome composition associated with OSCC. Although a direct causality has not been proven, individual members of the oral microbiome are capable of promoting various tumorigenic functions related to cancer development. Two prominent oral pathogens, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* can promote tumor progression in mice. *P. gingivalis* infection has been associated with oro-digestive cancer, increased oral cancer invasion, and proliferation of oral cancer stem cells. The microbiome can influence the evolution of the disease by directly interacting with the human body and significantly altering the response and toxicity to various forms of cancer therapy. Recent studies have shown an association of certain phylogenetic groups with the immunotherapy treatment outcomes of certain tumors. On the other side of the coin, recently it has been a resurgence in interest on the potential use of bacteria to cure cancer. These kinds of treatments were used in the late nineteenth and early twentieth centuries as the first line of defense against cancer in some hospitals but later displaced by other types of treatments such as radiotherapy. Currently, organisms such as *Salmonella typhimurium* and *Clostridium* spp. have been used for targeted strategies as potential vectors to treat cancer. In this review, we briefly summarize our current knowledge of the role of the oral microbiome, focusing on its bacterial fraction, in cancer in general and in OSCC more precisely, and a brief description of the potential use of bacteria to target tumors.

Keywords: oral microbiome, cancer, oral squamous cell carcinoma, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, immunotherapy, bacteria-mediated tumor therapy

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THE HUMAN MICROBIOME AND CANCER. AN OVERVIEW

About 30 trillion bacterial cells are living in or on every human. That is around one bacterium for each cell in the human body (1). These microorganisms are on the whole known as the microbiome. Since the completion of the Human Microbiome Project (2), we have witnessed an increased interest in the role that the human microbiome plays in human health, many studies have linked changes in microbial communities to systemic conditions such as allergies, diabetes, inflammatory bowel disease, and atherosclerosis (3–7). Among the systemic conditions influenced by the microbiome, cancer has not been an exception. We have learned that chronic infections

contribute to carcinogenesis, with approximately 13% of the global cancer burden being directly attributable to infectious agents (8).

Many viruses promote cancer through well-described genetic mechanisms. Around 10–15% of human cancers worldwide are caused by seven human viruses, which include Epstein-Bar Virus (EBV), Hepatitis B Virus (HBV), Human T-lymphotropic virus-I (HTLV-I), Human papillomaviruses (HPV), Hepatitis C virus (HCV), Kaposi's sarcoma herpesvirus (KSHV) and Merkel cell polyomavirus (MCV) (9). However, the first evidence that bacteria were directly involved in cancer development did not come until the 1980s with the work of Marshall and Warren (10). When they presented their results, entrenched was the belief that lifestyle caused ulcers that it was difficult for them to convince the scientific world of *Helicobacter pylori*'s role in gastric cancer (**Figure 1**). To provide even more conclusive evidence, in 1985, Marshall deliberately infected himself with the bacterium and established his stomach illness. Since then, it has been firmly proven by many researchers worldwide that *H. pylori* cause more than 90% of duodenal ulcers and up to 80% of gastric ulcers, and has been classified as a class I carcinogen by the World Health Organization due to its ability to promote stomach cancer after chronic infection (11–13). Disease-promoting and cancer-promoting effects of pathogens often depend on virulence factors. In *H. pylori*, strains expressing the virulence factors cytotoxin associated gene A (CagA) or vacuolating cytotoxin A (VacA), exemplify the role of virulence factors by increasing inflammation, and cancer rates (14).

An emerging concept in cancer biology implicates the microbiome as an influential environmental factor modulating the carcinogenic process. The idea that inflammation promotes carcinogenesis was first postulated more than 150 years ago by the German pathologist Virchow (15). The link between chronic inflammation and cancer is now well established (16–18). This association has recently experienced a renewed interest with the recognition that members of the human microbiome can be responsible for the chronic inflammation observed in a wide variety of cancers (19). Increasing evidence shows the association of changes in the human microbiome with certain types of cancer (20–22). Studies in germ-free animals have revealed evidence for tumor-promoting effects of the microbiome in spontaneous, genetically-induced, and carcinogen-induced cancers in various organs (23).

There is strong epidemiological evidence that other bacterial species are associated with cancer development, most likely induced by creating a pro-inflammatory micro-environment (24) or suppressing the immune response (25). Among the species of bacteria that have been directly linked to the development of cancer *is Salmonella enterica* subsp. *enterica* sv. *Typhi* (*S. Typhi*) and gallbladder cancer (26–28), *Streptococcus bovis* and colon cancer (29–31), and *Chlamydia pneumoniae* with lung cancer (32–36). The most persuasive epidemiological evidence of bacterial oncogenic potential, aside from *H. pylori*, concerns *S. Typhi*. However, the evidence for *S. bovis* and *C. pneumoniae* and *Fusobacterium nucleatum* is less conclusive, a meta-analysis on the association of those organisms with increased risk of cancer have shown either different or weak associations (33, 37–42).

Although gallbladder carcinoma (GBC) is rare in western countries, there is a high incidence in countries with endemic *S. Typhi* infections such as South America and parts of Africa and Asia, particularly India and Pakistan (43). The first epidemiological association was found by Welton et al. in 1979. In that paper, they analyzed 471 deceased typhoid carriers, registered by the New York City Health Department between 1922 and 1975, and matched with 942 controls for sex, age at death, year of death, the borough the carrier died, and where they were born. The results show that chronic typhoid

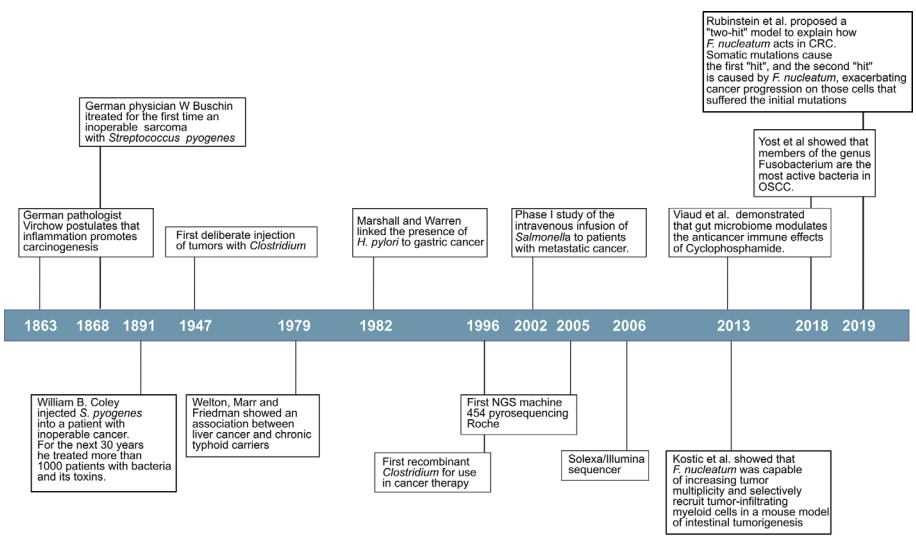

FIGURE 1 | Timeline: Some significant discoveries and events in cancer microbiome research.

carriers die of hepatobiliary cancer six times more often than the matched controls (44). Two more recent meta-analyses confirmed these initial results. In the first study by Koshiol et al., they performed a case-control and a meta-analysis of more than 1,000 GBC cases, and in both cases, they found a positive association between *S. Typhi* and GBC (45). In the second meta-analysis by Nagaraja and Eslick, they selected 17 studies for their analysis, most of them from India and China. The highest incidence of gallbladder cancer (GBC) occurs in India, contributing to about 10% of the global GBC cases (46). When Nagaraja and Eslick performed a subgroup analysis according to region, they found a significant association between *S. Typhi* carrier state and carcinoma of the gallbladder based on detection methods of *S. Typhi* antibody levels and culture (47). A possible mechanism has been proposed that explains the link between gallbladder carcinoma and infection by *S. Typhi*. In predispose mice, organoids and tissue culture with mutations in TP53 (tumor suppressor gene), and enhanced c-MYC (oncogenes) expression translocated bacterial effector molecules SopE, SopE2, SopB (Salmonella outer proteins), and SptP (*Salmonella* protein tyrosine phosphatase), from the *Salmonella* pathogenicity island 2 (SPI-2), activate the protein kinase B (Akt), or MAPK inhibitors, prevented mouse embryonic fibroblast transformation (48).

The previous examples refer to the link between specific organisms and carcinogenesis; however, microbes that trigger transformation events in host cells are rare. It has been demonstrated that in some cases, the tumorigenic process is not the result of the activities of a specific organism but rather the result of an instability in the composition of the bacterial communities or dysbiosis, often associated with inflammatory disorders such as colitis or periodontal disease. In mouse models, it has been shown that a dysbiotic community can lead to the development of colorectal cancer (49, 50).

The shift from a eubiotic community, with low cancer risk, to a dysbiotic community, with increased cancer risk, is the result of changes in environmental conditions and associated with metabolic responses in the host that modulate the progression of cancer (51). Dysbiosis of the oral microbiome could influence cancer outcomes by different mechanisms. Two common mechanisms that could have severe implications in the development of the disease are chronic inflammation and the synthesis of metabolites that could induce mutations (52).

The Oral Microbiome and Cancer

There were reports of a correlation between periodontitis and leukemia as early as the late 1940s early 1950s (53, 54). Since those pioneer studies, there is mounting evidence of the correlation between periodontal disease and various cancers. Several meta-analyses have confirmed the suspicion that periodontal disease should be considered as a risk factor in several types of cancers. In fact in a meta-analysis by Corbella et al., they found that a statistically significant association was found for all cancers studied, both combined and individually (digestive tract, pancreatic, prostate, breast, corpus uteri, lung, hematological, esophageal/oropharyngeal and Non-Hodgkin lymphoma) (55). Never smokers population with periodontal disease has a higher risk of developing hematopoietic and lymphatic cancers (55, 56). Pancreatic, lung, and colorectal cancers also show a positive correlation with periodontal disease (55, 57–60). In the case of breast cancer, the evidence shows a more modest positive association between periodontal disease and breast cancer (61). Finally, the most expected correlation would be with oral cancers, and indeed there is a clear positive correlation between periodontal disease and oral cancers (55, 59, 60). Edentulism has been positively correlated with pancreatic cancer (58) but not within colorectal cancer (62). Interestingly, in the study where authors did not find associations of edentulism and colorectal cancer (CRC), they also found no correlation between edentulism and periodontitis (62). One of these meta-analyses looked at not only a correlation between periodontal disease and cancer but also correlations with particular organisms (60). The results of that meta-analysis indicated that periodontal bacterial infection increased cancer incidence and was associated with poor overall survival, disease-free survival, and cancer-specific survival. Subgroup analysis indicated that the risk of cancer was associated with *Porphyromonas gingivalis* and Prevotella intermedia infection but not *Tannerella forsythia*, *Treponema denticola*, *Aggregatibacter actinomycetemcomitans*, and *F. nucleatum* infection (60).

In the case of pancreatic cancer, a potential mechanism has been proposed (57). The innate immune response to pathogenic bacteria that results in inflammation also has been linked with pancreatic carcinogenesis. Lipopolysaccharide, a component in the outer membrane of Gram-negative bacteria, such as, *P. gingivalis*, triggers an innate immune response that involves recognition by Toll-like receptor 4 (TLR4), which stimulates both myeloid differentiation primary response 88 (MyD88) dependent and MyD88-independent pathways that then activate the nuclear factor κB pathway and results in the release of pro-inflammatory cytokines. Interestingly, TLRs seem to have a role in pancreatic cancer development, and TLR4 is explicitly highly expressed in human pancreatic cancer but not a healthy pancreas. In the murine models, the TLR4/MyD88 pathway can trigger protection from pancreatic cancer development or acting to promote inflammation and pancreatic cancer development (63). LPS was shown to drive pancreatic carcinogenesis, as was the blockade of the MyD88-dependent pathway (via a dendritic cell-mediated deviation to T_H2), whereas blockade of TLR4 (via TRIF, TIR-domain-containing adapter-inducing interferon-β) and blockade of the MyD88 independent (via TRIF) were protective against pancreatic cancer (63). Although more empirical evidence is needed, similar mechanisms implicating inflammatory response of the innate immune system may be implicated in other types of cancer.

Oral Microorganisms and Cancers Outside the Oral Cavity

One characteristic of the oral microbiome that distinguishes from other body sites is that many oral microorganisms that are considered commensals in the oral cavity are commonly

associated with various cancers in distant organs (**Table 1**). The associations of oral organisms and distant cancers occur in two categories. The first appears in certain types of cancer where no oral microbes are directly involved in the tumor's pathogenesis, but there is a consistent change in the composition of the oral microbiome associated with cancer, thus with the potential use of those changes as biomarkers of cancer (71, 86). The second kind of association is the association of organisms involved in tumorigenesis, such as *F. nucleatum* association with CRC (88, 89, 100).

Dysbiosis of the oral microbiome has been associated with a wide variety of cancers (**Table 1**). There are three types of associations between microbiome and disease that have been described as potential biomarkers in cancer: increase or decrease in numbers of individual organisms, use of models of several organisms as predictors and assessing their performance, and finally changes in diversity indexes that give an overall picture of the behavior of the community.

As examples of specific organisms as biomarkers, an increase in *F. nucleatum* has been linked to CRC (88), and *P. gingivalis* (69) and *Fusobacterium* spp. (70) have been linked to pancreatic cancer. Additional examples of links of specific bacteria and oral organisms are presented in **Table 1**. Another approach in the search for biomarkers of cancer is the use of complex models of several organisms that maximize the area under the Receiver Operating Characteristics (ROC) curve or Area Under Curve (AUC). ROC shows how well a model can discriminate or separate the cases and controls, and AUC has a value between

TABLE 1 | Oral organisms associated with distant tumors.

Cancer	Organisms	Sample type	Reference
Esophageal cancer	Increase of *T. forsythia* and *P. gingivalis*	Oral rinse	(64)
Esophageal cancer	*Streptococcus anginosus*, *S. mitis*, *Treponema denticola*	Saliva	(65)
Esophageal cancer	3 taxon model: *Lautropia*, *Streptococcus*, and an unspecified genus of the order *Bacteroidales.* (AUC = 0.94)	Oral swab	(66)
Esophageal cancer	Overall decreased microbial diversity in cancer patients	Saliva	(67)
Pancreatic cancer	*Porphyromonas gingivalis, Aggregatibacteractinomycetemcomitans*	Oral rinse	(68)
Pancreatic cancer	*Porphyromonas gingivalis*	Blood (antibodies)	(69)
Pancreatic cancer	*Fusobacterium* spp.	Tissue from pancreatic ductal adenocarcinoma	(70)
Pancreatic cancer	2 taxon model: *Streptococcus mitis* and *Neisseria elongata.* (AUC =0.90)	Saliva	(71)
Pancreatic cancer	Significative higher ratio of *Leptotrichia*to *Porphyromonas* was found in cancer patients.	Saliva	(72)
Pancreatic cancer	Association with β-diversity and *Haemophilus*	Saliva	(73)
Pancreatic cancer	*Fusobacterium* spp.	Tissue samples, swabs, stool	(74)
Pancreatic cancer	*Streptococcus thermophilus* higher in cancer*, and Haemophilus parainfluenzae* and *Neisseria flavescens* lower in cancer	Saliva	(75)
Pancreatic cancer	*Haemophilus, Porphyromonas, Leptotrichia* and *Fusobacterium* could distinguish cancer patients from healthy subjects	Tongue coating microbiota	(76)
Hepatic cancer	*Fusobacterium* and *Oribacterium*. Increase in diversity.	Tongue coat	(77)
Lung cancer	*Capnocytophaga* sp., *Veillonella* sp.	Saliva	(78)
Lung cancer	*Streptococcus* and *Veillonella*	Airway brushings	(79)
Lung cancer	*Sphingomonas* and *Blastomonas*	Saliva	(80)
Lung cancer	*Streptococcus* and *Veillonella*	Saliva	(81)
Colorectal cancer	*T. denticola* and *Prevotella* sp. oral taxon 313	Oral rinse	(82)
Colorectal cancer	*Fusobacterium* sp., *Porphyromonas* sp.	Stool	(83)
Colorectal cancer	*Fusobacterium sp.*	Colorectal cancer tissues	(84)
Colorectal cancer	*Lactobacillus* and *Rothia*	Oral rinse	(85)
Colorectal cancer	*Streptococcus* and *Prevotella* spp.	Oral swabcolonic mucosae and stools, colorectal polyps or controls	(86)
Colorectal cancer	*Fusobacterium sp.*	Tissue and stool samples	(87)
Colorectal cancer	*Fusobacterium nucleatum*	Colorectal tissue biopsies	(88)
Colorectal cancer	*Fusobacterium sp.*	Colorectal tissue biopsies	(89)
Colorectal cancer	*Fusobacterium sp.*	Colorectal tissue biopsies	(21)
Colorectal cancer	*Fusobacterium sp., Lactococcus sp.*	Colorectal tissue biopsies	(90)
Digestive tract cancer	*Actinomyces odontolyticus, Steptococcus parasinguinis, Corynebacterium* spp.*, Neisseria* spp.,TM7[G-1] sp., *Porphyromonas gingivalis, Fusobacterium nucleatum, Neisseria elongata and Streptococcus sanguinis*	Saliva	(91)
Colorectal cancer	*Fusobacterium nucleatum, Parvimonas micra*, and *Peptostreptococcus stomatis*	Colon tissue	(92)
Colorectal cancer	*Peptostreptococcus stomatis, Fusobacteriumnucleatum, Parvimonas* spp.	Meta-analysisfecal samples	(93)
Gastric cancer	Overall diversity of tongue coating microbiota was reduced	Tongue coating	(94)
Gastric cancer	Overall increased microbial diversity in cancer patients	Saliva and plaque samples	(95)
Gastric cancer	6 bacterial clusters were identified to distinguish cancer patients from controls. (cluster 6 had AUC = 0.76)	Tongue coating	(96)
Breast cancer	*Corynebacterium, Staphylococcus, Actinomyces*, and *Propionibacteriaceae*	Urine	(97)
Breast cancer	*Fusobacterium, Atopobium, Gluconacetobacter, Hydrogenophaga* and *Lactobacillus*	Breast tissue	(98)
Breast cancer	*Coriobacteriaceae*	Oral rinse	(99)

0.50 and 1.0, indicating no discrimination and perfect discrimination, respectively (101). Thus, and AUC of 0.9 would indicate that the probability of discriminating a case vs. control is 90%. In **Table 1**, we show three examples of combined taxons to distinguish controls vs. cancer samples: a three taxon model of *Lautropia*, *Streptococcus* and an unspecified genus of the order *Bacteroidales*, with AUC =0.94, has been associated with esophageal cancer (66), a two taxon model, *Streptococcus mitis* and *Neisseria elongata* with an AUC of 0.90, associated with pancreatic cancer (71) and a bacterial cluster associated with gastric cancer with AUC of 0.76 (96). Finally, another approach to the use of biomarkers in differentiating case vs. control samples is using changes in biodiversity indicators as a proxy of changes in the microbiome that could be linked to the status of the sample, independently of what organisms are present or absent. We have also presented examples of this approach in **Table 1**. For instance, Chen et al. observed an overall decrease of microbial diversity in the saliva of esophageal cancer patients (67), while an overall increase in diversity in saliva and plaque from gastric cancer patients was observed by Sun et al. (95).

Except for CRC, where *Fusobacterium* spp. are ubiquitous, in general, there is little overlapping in the biomarkers identified in the different studies (**Table 1**), probably due to the different type of samples and methods of analysis used to identify the composition of the community.

Pancreatic cancer has been one of the most widely studied types of cancer in seeking association between changes in the oral microbiome and disease (68–76). Although the communities vary depending on the study, *Porphyromonas* and *Fusobacterium* genera were associated with cancer in most studies (**Table 1**). Likewise, in the case of lung cancer, the genus *Veillonella* appeared associated with the majority of studies (**Table 1**).

CRC has been an exception in that *Fusobacterium* spp. have been consistently associated with this type of cancer, both as a biomarker in the oral cavity (91) and more importantly, in stool and biopsies from tumors samples (21, 84, 87–90, 92, 93), indicating a possible direct effect on the progression of the disease. A more direct indication of the clinical relevance of *F. nucleatum* in CRC has been presented in a recent paper where the authors show that in mice with colorectal tumors, oral or intravenous administration of dextran nanoparticles covalently linked to azide-modified phages that inhibit the growth of *F. nucleatum* significantly augments the efficiency of first-line chemotherapy treatments of CRC (102). For all these reasons, most focus has been placed on studying the role of *F. nucleatum* in CRC. Using arbitrarily primed PCR (AP-PCR) to identify isolates at the strain level, Komiya et al. demonstrated that the strains present in CRC samples were identical to strains isolated from the saliva of CRC patients, supporting the oral origin of *F. nucleatum* in the intestine of CRC patients (103).

In 2012 Kostic et al., using genomic analysis, identified *Fusobacterium* sequences as enriched in colon carcinomas; they did not prove a causal relationship between *Fusobacterium* and colorectal cancer was the first indication of its potential importance in CRC (21). One year later, Kostic et al., using the mouse model of intestinal tumorigenesis Apc(Min/+), showed that *F. nucleatum* was capable of increasing tumor multiplicity and selectively recruit tumor-infiltrating myeloid cells, which can promote tumor progression. Tumors from Apc(Min/+) mice exposed to *F. nucleatum* exhibit a pro-inflammatory expression signature shared with human fusobacteria-positive colorectal carcinomas. *F. nucleatum* generates a pro-inflammatory microenvironment conducive to colorectal neoplasia progression by recruiting tumor-infiltrating immune cells (87). This same year Rubinstein et al. proposed a possible mechanism by which *F. nucleatum* induces tumorigenesis (104). *F. nucleatum* encodes several adhesins for interspecies interactions, but so far, only one, FadA (adhesion protein FadA), has been identified as binding to host cells (105). FadA is not only an adhesin but also an invasin required for binding and invasion of both healthy and cancerous host cells and binds to cell-junction molecules, the cadherins (105). In Rubinstein et al. model, FadA binds to E-cadherin activating β-catenin signaling (104). The activation of β-catenin signaling in colorectal cancer is mediated *via* a TLR4/P-PAK1 (p21-activated kinases) cascade (106). Loss of E-cadherin-mediated-adhesion characterizes benign lesions' transition to invasive, metastatic cancer (107, 108).

In a more recent article, Rubinstein et al. expanded their model to include Annexin A1, a previously unrecognized modulator of Wnt/β-catenin signaling, which is a crucial component through which *F. nucleatum* exerts its stimulatory effect. Annexin A1 is expressed explicitly in proliferating colorectal cancer cells and involved in the activation of Cyclin D1 (109). Over-expression of cyclin D1 has been linked to the development and progression of cancer (110). Based on their results, Rubinstein et al. proposed a "two-hit" model to explain how *F. nucleatum* acts in CRC. Somatic mutations cause the first "hit", and the second "hit" is caused by *F. nucleatum*, exacerbating cancer progression on those cells that suffered the initial mutations (109).

The Microbiome and Oral Cancer

Head and neck cancer was the seventh most common cancer worldwide in 2018 (890,000 new cases and 450,000 deaths) (111). Oral squamous cell carcinomas (OSCC), the most frequent malignancies in the oral cavity, represented 2% of all cancers worldwide (354,864 cases and 177,384 deaths) (111). In the United States alone, it is expected that a total of 52,260 new cases of oral and pharynx cancer will occur in the year 2020 (112). The financial cost of treating oral and oropharyngeal cancer may be the highest of all cancers in the United States (113). Additionally, an essential factor in OSCC mortality is the high level of recurrence after treatment. Several studies, with many cases, have shown that the overall recurrence rate was approximately 30% (114–116). Recurrence rate in OSCC is high. In the first 36 months, the recurrence rates range from 70 to 92% of cases (114, 117–119). Interestingly the observed 5-year survival after recurrence varies depending on the moment in which recurrence occurs. If recurrence occurs within the 18 months after treatment, the rate of survival ranges between 20.5 to 27.55%, but if recurrence occurs afterward, the rate of survival increases to 38.1% to 42.3% (114, 120).

Despite advances in our knowledge of the causes and risk factors associated with OSCC, survival rates for oral cancers, including OSCC have not improved substantially in the last forty years, emphasizing that new means of early detection and treatment are urgently needed.

Viruses have long been associated with the risk of developing OSCC. The etiological role of human papillomavirus (HPV) in OSCC has been widely researched for more than three decades. Several meta-analyses have demonstrated that infection by HPV increments the risk of OSCC by up to 3-fold (121, 122). The prevalence of HPV among OSCC patients is around 25% (121, 123, 124); however, there is a geographical component with the highest HPV prevalence in Africa and Asia, notably among Chinese studies from provinces with high OSCC incidence rates (123). Other viruses have been identified in OSCC samples, as individual infections or in co-infection with HPV. Still, their role in the disease is unclear (124–127). Despite its importance, HPV-unrelated OSCC cases account for the vast majority of oral cancer cases. Other environmental factors should play an essential role in OSCC development and progression; among them, the microbiome's role has just begun to be considered a risk factor.

As mentioned previously, periodontitis has been linked to various types of cancers, including esophagus/oropharyngeal cancers. **Table 2** presents some of the studies that linked bacteria's presence to cancer of the oral cavity and pharynx. Several studies have found that the risk of developing OSCC may increase with periodontal disease (55, 142, 143), signaling a possible role of inflammation caused by the microbiome with oral cancer. Periodontitis is a typical example of an infectious disease causing chronic inflammation in the oral cavity (144, 145). Thus several systematic reviews of the literature showed that periodontal disease increases the risk of oral cancer even after adjusting for significant risk factors (146, 147).

Moreover, expression of pro-inflammatory cytokines in periodontal disease such as IL-1 and TNF-α has been linked to microbial triggered carcinogenesis (19). In a study comparing the microbiome of gingival squamous cell carcinoma (GSCC) with periodontitis microbiome, members of the genera *Fusobacterium*, *Peptostreptococcus*, and *Prevotella* were more abundant in cancerous, periodontal tissues. In contrast, saliva or soft mucosa harbored more periodontal health-related bacteria (148).

Most studies on the role of the human microbiome on cancer have focused on describing microbial communities present in specific samples or the immunological response of the host to the bacterial challenge. The oral microbiome has been proposed as a diagnostic indicator of oral cancer; however, as in other types of

TABLE 2 | Oral organisms in oropharyngeal cancers.

Cancer	Organisms	Sample type	Reference
Head and neck squamous cell carcinoma (HNSCC)	*Streptococcus sp. and Lactobacillus sp.*	Saliva	(128)
HNSCC	*Streptococcus anginosus*	Tissue	(129)
HNSCC	*Fusobacterium sp.*	Meta-analysis	(38)
Oral squamous cell carcinoma (OSCC)	*Streptococcus anginosus*	Tissue	(130)
OSCC	*Capnocytophaga gingivalis, Prevotella melaninogenica, Streptococcus mitis*	Saliva	(131)
OSCC	*Bacillus, Enterococcus, Parvimonas,Peptostreptococcus, Slackia*	Saliva	(132)
OSCC	*Streptococcus sp. 058, S. salivarius, S. gordonii, S. parasanguinis, Peptostreptococcus stomatis, Gemella haemolysans, G.morbillorum, Johnsonella ignava*	Tissue	(133)
OSCC	*Parvimonas* increased in OSCC, *Actinomyces* reduced in OSCC	Tissue	(134)
OSCC	*Fusobacterium periodonticum, Parvimonas micra, Streptococcus constellatus, Haemophilus influenza*, and *Filifactor alocis*	Oral rinse	(80)
OSCC	*Fusobacterium nucleatum, Prevotella intermedia, Aggregatibactersegnis, Capnocytophaga leadbetteri, Campylobacter rectus,Catonella morbi, Corynebacterium matruchotii, Gemella morbillorun, Granulicatella adjacens, Granullicatella elegans, Peptococcus* sp., *Peptostreptococcus stomatis, Porphyromonas catoniae* and *Streptococcus oralis*	Tissue	(135)
OSCC	*Fusobacterium, Dialister, Peptostreptococcus, Filifactor, Peptococcus, Catonella* and *Parvimonas*	Swabs	(136)
OSCC	*Micrococcus luteus, Prevotella melaninogenica, Exiguobacterium oxidotolerans, Fusobacterium naviforme, Staphylococcus aureus, Veillonella parvula, Prevotella sp. (oral clone BE073 phylotype), Rothia mucilaginosa, Streptococcus salivarius, Actinomyces odontolyticus, Moraxella osloensis, Prevotella veroralis, Propionibacterium acnes, Atopobium parvulum, Streptococcus parasanguinis, Veillonella dispar, Streptococcus mitis/oralis*	Tissue	(137)
Gingival squamous cell carcinoma	*P. gingivalis*	Paraffin embedded samples	(138)
Oral mucosal cancer	*Streptococcus intermedius, S. constellatus, S. oralis, S. mitis, S. sanguis, S. salivarius, Peptostreptococcus sp.*	Lymph nodes	(139)
Keratinizing squamous cell carcinoma	*Veillonella* sp., *Fusobacterium* sp., *Prevotella* sp., *Porphyromonas* sp., *Actinomyces* sp., *Clostridium* sp., *Haemophilus* sp., *Streptococcus sp.*, and *Enterobacteriaceae*	Swabs	(140)
Potentially malignant oral leukoplakia	*Fusobacterium, Leptotrichia, Campylobacter* and *Rothia*	Swabs	(141)

cancer, the search for possible biomarkers of oral cancer and, most specifically, OSCC has not produced conclusive results (52). Different organisms have been shown to increase in OSCC samples, as a few examples: *Capnocytophaga gingivalis*, *Prevotella melaninogenica*, and *S. mitis* (131); *F. nucleatum* (149); *Pseudomonas aeruginosa* (150); *Campylobacter concisus*, *Prevotella salivae*, *Prevotella loeschii*, and *Fusobacterium* oral taxon 204 (151); genera *Fusobacterium*, *Dialister*, *Peptostreptococcus*, *Filifactor*, *Peptococcus*, *Catonella*, and *Parvimonas* (136); and *Prevotella oris*, *Neisseria flava*, *Neisseria flavescens/subflava*, *F. nucleatum ss polymorphum*, *Aggregatibacter segnis*, and *Fusobacterium periodonticum* (152).

Yang et al. studied the progression of the microbiome during cancer's progression from the early to the late stage and found a significant increase of *Fusobacteria.* At the species level, they found that *F. periodonticum*, *Parvimonas micra*, *Streptococcus constellatus*, *Haemophilus influenza*, and *Filifactor alocis* were associated with OSCC, and they progressively increased in abundance from stage 1 to 4 (80).

Direct evidence of the role of the microbiome in OSCC was presented by Stashenko et al. were using a germ-free mouse model a 4-nitroquinoline-1 oxide (4NQO)-induced carcinogenesis the authors observed a significant increase on the number of tumors and their size when the mice were inoculated with two different microbiomes vs. inoculated controls. The microbiomes used came from the tongue of a healthy mouse and the other from the tumor lesion of a diseased mouse, and *Pasteurella* was the dominant genus in both groups (153).

The carcinogenic potential of periodontal pathogens has also been described in several studies. *P. gingivalis*, one of the essential periodontal pathogens, increase the invasiveness of oral cancer cells and resistance to chemotherapeutic agents (154–156). Using a 4NQO-induced mouse model of oral cancer, the inoculation of *P. gingivalis* promoted tumor progression by invading precancerous lesions and recruiting the myeloid-derived suppressor cells by expressing chemokines such as C-C motif ligand 2 (CCL2) and chemokine (C-X-C motif) ligand 2 (CXCL2), and cytokines such as IL-6 and IL-8 (157). *P. gingivalis* also induces expression of the ZEB1 transcription factor, which controls the epithelial-mesenchymal transition. Interestingly, the up-regulation of ZEB1 appears to be controlled by FimA, a major virulence factor involved in adhesion and cellular invasion (158). The infection of *P. gingivalis* increases the expression of mesenchymal markers, including vimentin and matrix metalloproteinase MMP-9 (132, 158, 159).

Utilizing a murine model of periodontitis-associated oral tumorigenesis, Binder Gallimidi et al. showed that chronic bacterial co-infection of *P. gingivalis* and *F. nucleatum* promotes OSCC *via* direct interaction with oral epithelial cells through Toll-like receptors, with an increase in expression of TLR2 in OSCC cells and IL-6 in both cells and the mouse model (160). A detailed review of all the studies where *P. gingivalis* has been associated with the development of OSCC has been recently published by Lafuente Ibáñez de Mendoza et al. (161).

F. nucleatum in esophageal cancer tissues has been associated with shorter survival, suggesting a prognostic biomarker's potential role. *F. nucleatum* might also contribute to aggressive tumor behavior through the activation of chemokines, such as chemokine (C-C motif) ligand 20 (CCL20) (162). Epithelial-mesenchymal transition (EMT) is the process by which epithelial cells acquire a mesenchymal-like phenotype. It has been proposed that EMT is responsible for compromising epithelial barrier function in the pathogenesis of several diseases, including OSCC. *F. nucleatum* infection of oral cell lines triggers EMT (163, 164). *F. nucleatum* triggers EMT *via* lncRNA/MIR4435-2HG/miR-296-5p/Akt2/SNAI1 signaling pathway and up-regulates mesenchymal markers, including N-cadherin, Vimentin, and snail family transcription repressor 1 (SNAI1) (164). The fact that infection by periodontal pathogens generates EMT features introduces the possibility that this process may be involved in the loss of epithelial integrity during periodontitis and may promote predisposition to malignant transformation through the EMT.

The metatranscriptome has recently begun to be used to analyze community-wide gene expression in the human microbiome (165–168). Measuring bacterial gene expression in the wild has been challenging. The half-life of mRNA is short, and mRNA in bacteria and archaea usually comprises only a small fraction of total RNA. Therefore large samples are needed to study expression, but obtaining such samples is not always possible. Additionally, working with non-model organisms presents the challenge of lacking most of the bioinformatic tools readily available for model organisms.

Using a metatranscriptome analysis of OSCC samples, Yost et al. found that *Fusobacteria* showed a statistically significantly higher number of transcripts at tumor sites, indicating a higher activity of this group of organisms in cancer. Moreover, when looking at tumor signatures of the oral microbiome, metabolic activities such as iron ion transport, tryptophanase activity, peptidase activities, and superoxide dismutase were over-represented in tumor samples when compared to the healthy controls (169).

Effect of the Microbiome in the Outcomes of Cancer Therapy

Despite its clinical importance, the human microbiome's effect on malignancy treatment is merely starting to be investigated. While organisms assume significant activities in keeping up human well-being, they are likewise engaged with the turn of events that lead to tumor development. There is now proof indicating that the microbiome can impact patient reactions to cancer treatment. The microbiome has been implicated in modulating cancer therapy's efficacy and toxicity, including chemotherapy and immunotherapy (170, 171). Moreover, preclinical data suggest that the microbiome modulation could become a novel strategy for improving the efficacy of immune-based therapies for cancer (172).

The gut microbiota has the potential to affect the ability of cancer therapy. The microbiota, when affected by dysbiosis, can profoundly influence both cancer pathogenesis and its

therapeutic outcome. In particular, the regulation of such a therapeutic outcome is firmly linked with the gut microbiota's capacity to process anti-cancer compounds and modify the host's immune response. These two effects combined may clarify the patient's microbiome composition's substantial participation in affecting the efficiency of both immunotherapy and chemotherapy.

The oral microbiome's role in the outcome of cancer treatments has not yet been evaluated. Only in two studies, oral organisms were identified as influencing the outcome of immunotherapies. The first of these studies showed that *F. nucleatum*, along with *Bacteroides fragilis* and *Escherichia coli* improved the survival of adoptive cell therapy (ACT) treated patients, probably by increasing cytokine production and T cell infiltration (173). The second study found that *Lactobacillus fermentum* attenuated the immune response in patients treated with CpG-oligonucleotides, which are short synthetic single-stranded DNA molecules containing unmethylated CpG dinucleotides, acting as agonists of Toll-like receptor 9 (TLR9), and leading to strong immunostimulatory effects (174).

EFFECTS ON CANCER CHEMOTHERAPY

A recent survey of *in situ* bacterial effects on frequently used chemotherapeutics suggests the profound influence of distinct bacteria species on the anti-tumor effect. Heshiki et al. examined the influence of the intestinal microbiome on treatment effects (175) in a heterogeneous cohort that comprised eight diverse malignancy types to recognize organisms with a collective effect on the immune response. It is revealed through human gut metagenomic examination that responder patients had inherently higher microbial diversity arrangements than non-responders. Moreover, by assessing the gut microbiome's job without precedent for a heterogeneous patient cohort with different kinds of malignant growth and anti-cancer medicines, Heshiki et al. found a worldwide microbiome signature that is autonomous of disease type and heterogeneity (175). Explicit species, *Bacteroides xylanisolvens*, and *Bacteroides ovatus* were decidedly connected with treatment results. Oral gavage of these responder microbes fundamentally expanded the adequacy of chemotherapy (erlotinib) and actuated the declaration of CXCL9 and IFN-γ in a murine lung cancer model. Also, oral gavage of explicit gut microbiome substantially expanded the impact of chemotherapy in mice, decreasing the tumor volume by 46% contrasted with the control.

This information recommends an anticipated effect of the microbiome's explicit constituents on tumor development and disease treatment results with suggestions for both visualization and treatment. Curiously, the microbiome can bolster the resistant framework in the battle against malignancy. For instance, cyclophosphamide (a medication used to treat leukemia and lymphomas) was found to impact the organisms living in the gut. These gut organisms reacted by advancing the production of resistant cells, which appears to improve cyclophosphamide adequacy (176). Among patients with hematologic malignancies, specific bacterial taxa are connected with the effectiveness of allogeneic hematopoietic stem cell transplantation (allo-HSCT) and reduced hazard for graft-versus-host disease (GVHD) succeeding treatment (177, 178). Besides, holding expanded measures of microorganisms having a place with the variety of *Blautia* was related to diminished GVHD lethality in this cohort and was affirmed in another independent cohort of 51 patients from a similar organization (177).

EFFECTS ON CANCER RADIOTHERAPY

Ionizing radiation therapy (RTX) was received by cancer patients that are genotoxic for tumor cells and might be curative for restricted cancers. The great worldview in radiation biology accepted that the cellular nucleus was the main objective of radiation and DNA harm was incited by direct testimony of vitality or creation of reactive oxygen species (ROS) through a radiation-induced separation of intracellular water molecules. Ionizing radiation, however, additionally incites non-targeted impact on non-irradiated cells, such as genomic unpredictability, systemic radio-adaptive retorts, inflammatory and immune reactivity, and bystander effect on nearby cells. Observer and foundational impacts are auxiliary to DNA harm and are intervened by the interruption of gap intersection proteins engaged with cell-cell interactions and by the arrival of extracellular intermediaries, together with cytokines, exosomes, ROS and nitric oxide (NO). Along these lines, comparably to the tissue harm related to contamination by microorganisms, radiations initiate the arrival of damage-associated molecular pattern (DAMP) pressure signals (179). The impacts of radiation are perplexing. It initiates both immunostimulant and immunosuppressive reactions and might be deficient in enacting a defensive anti-cancer invulnerable reaction (180). It very well may be speculated that the gut microbiome likewise satisfies a job in the immunostimulatory impacts of RTX. The viewed fluctuations in microbiome arrangement at epithelial surfaces in patients and mice rewarded with RTX have been proposed to contribute to the pathogenesis of bone marrow failure, colitis, looseness of the bowels, oral mucositis and enteritis (181). RTX persuades apoptosis in the breach of the intestinal barrier, intestinal crypts, and modifications in the microbiome conformation (182).

Studies have shown that the intestinal microbiome has a significant effect on total body irradiation. Fewer endothelial cells of the intestinal mucosa are derived through irradiation into apoptosis and prompt less lymphocyte invasion in germ-free mice than in conventional mice (183). This finding shows that gut commensals can assume a harmful role in protecting the enteric harmfulness of total body irradiation (TBI) in germ-free mice.

EFFECTS ON CANCER IMMUNOTHERAPY

Ongoing examinations have featured the significance and possible effect of organisms on ailment recuperation of

immunotherapy. Strikingly, the microbiome can bolster the safe framework in the battle against malignant growth (176). Microorganisms have been appeared to advance cancer growth improvement by instigating inflammation. This provocative reaction can, likewise, beneficially affect malignancy treatment. A few treatments, such as CpG-oligonucleotide immunotherapy, rely on inflammation (174).

In an examination performed by Iida et al., it was seen that mice treated with anti-toxins did not react to platinum chemotherapy or CpG-oligonucleotide insusceptible treatment contrasted with mice with flawless gut microorganisms of subcutaneous tumors. These outcomes recommend that gut microbiome improves the impacts of treatments that are subject to aggravation. Desirable reactions to cancer therapy require an unblemished commensal microbiome that intervenes in its belongings by regulating myeloid-inferred cell capacities in the tumor microenvironment. These discoveries underscore the significance of the microbiome in the outcomes of sickness treatment (174).

In most patients treated with traditional cancer therapies, tumors become resistant to therapy, and the chances of tumor recurrence are high (184). Immunotherapy approaches have shown potential in treating hematopoietic (185, 186) and solid cancers (187–189). However, the efficacy of immunotherapy is still limited by the variability of the immune response in different patients and the different susceptibility of tumor types (190, 191). The emerging knowledge of the ability of the gut microbiome to modulate the response to immunotherapy offers new possibilities to improve its efficacy by targeting the microbiome.

Utilization of Check Point Inhibitors (ICIs) has revolutionized cancer treatment across multiple cancer types and has gotten the first since forever FDA endorsement of a tumor agonistic agent in tumors with microsatellite instability (MSI) (192). The most generally utilized ICIs are monoclonal antibodies that focus on the customized cell demise protein (PD-1), its ligand (PD-L1), or the cytotoxic T-lymphocyte antigen 4 protein (CTLA-4). **Table 3** shows the microbiome in malignant growth patients dealt with immune checkpoint inhibitors (ICIs). It was proposed by Sivan (197) that *Bifidobacterium*, a particular taxon of microbial commensals, armed anti-tumor resistance and raised the viability of PD-L1 blocking treatment. They additionally recommended that microbiome could modify the anti-tumor invulnerability as well as the reaction to PD-L1 inhibitors. Routy et al. investigated the relationship of dysbiosis with epithelial tumors to comprehend whether synchronous utilization of anti-infection agents creates essential protection from ICIs in mice and patients. Their outcomes indicated that the anti-tumor impact was undermined in antibiotic (ATB) treatment group, with progression-free survival (PFS) and overall survival (OS), being fundamentally shorter contrasted with that of the control group, showing that ATB could be utilized as a prescient marker for estimating ICIs obstruction. Additionally, utilizing the shotgun sequencing for quantitative metagenomics of the fecal example, *Enterococcus hirae* and *Akkermansia muciniphila* were demonstrated to be altogether plentiful in patients with best clinical reaction to ICIs (PFS > 3 months) (196).

Naidoo et al. established a connection between the gut microbiome and remedial results after the clinical examination of Chinese patients with cutting edge non-little cell lung carcinoma treated with PD-1 ICIs treatments. As indicated by their outcomes, reacting patients held greater assorted variety and stable arrangement of the natural gut microbiome during treatment and had drawn-out PFS altogether. In detail, *Prevotella copri*, *Bifidobacterium longum*, and *Alistipes putredinis* were improved in responders, though *Ruminococcus* spp. was found mainly in non-reacting patients. As expected, in the periphery blood of responding patients, a more noteworthy recurrence of natural killer cell and memory CD8+ T cell subgroups was seen (198).

A consortium of 11 bacterial strains was isolated by Tanoue et al. from healthy human donor feces that can thrive in the intestine, increasing interferon-γ-producing CD8 T cells. The colonization of mice improved immune checkpoint inhibitors' therapeutic effectiveness with these 11-strain mixtures in syngeneic tumor models. All these strains act together in a way that is subject to significant histocompatibility (MHC) class Ia molecules and $CD103^+$ dendritic cells. Primarily the 11 strains represent rare, low-abundance components of the human microbiome, and subsequently, have incredible potential as extensively powerful biotherapeutics (199).

Then again, immunotherapy viability has all the earmarks of being intensely impacted by gut microbiome confirmation. Oral organization of probiotics, for example, *A. muciniphila* (196) and *Bifidobacterium* species (197) or fecal microbiota transplantation (FMT) (200) from treatment-responsive patients, considerably upgraded the PD1-based immunotherapy and canceled tumor outgrowth, robotically through the enlarged dendritic cell and T cell reaction (197). Even though these examinations are not utilizing colorectal cancer (CRC) models, seeing how gut microbiome adjusts strong reaction might be necessary to encourage positive remedial results in CRC patients getting

TABLE 3 | Characteristics of the microbiome in cancer patients treated with immune check point inhibitors (ICIs).

Cancer	Microbes related with response to ICI treatment	References
Metastatic melanoma	*B. fragilis* and/or *B. thetaiotaomicron, Burkholderiales* species	(193)
Metastatic melanoma	*Faecalibacterium* genus and other Firmicutes	(194)
Metastatic melanoma	*Enterococcus faecium*, *Collinsella aerofaciens*, *Bifidobacterium adolescentis*, *Klebsiella pneumoniae*, *Veillonella parvula, Parabacteroides merdae*, *Lactobacillus* species, and *Bifidobacterium longum*	(195)
Non-small cell lung carcinoma	*Ruminococcaceae*, *Faecalibacterium*, specifically *Ruminococcae, Alistipes*, and *Eubacterium* species	(196)
Renal cell carcinoma	*Ruminococcaceae*, *Faecalibacterium*, specifically *Ruminococcae, Alistipes*, and *Eubacterium* species	(196)

immunotherapy, or even to conquer opposition-held by non-responders. To our best information, no clinical preliminaries assessing gut microbiome control and treatment viability are distributed right now (201). A couple of clinical preliminaries are started and now at the selecting stage (**Table 4**) in light of Fong et al. (201). It stays dark whether these preclinical discoveries can be effectively meant clinical application.

The microbiome, aside from the balance of adequacy, may likewise foresee the susceptibility to immunotherapy-associated unfavorable events. While ICIs have given way to overawed immunological resilience to tumors, the danger of auto insusceptibility in healthy tissues is a critical restriction in their utilization. In patients on anti-CTLA-4, the immune-related adverse events (irAEs) happen all the more generally contrasted with those taking anti-PD-1/PD-L1, and when these mediators are used in amalgamation, the frequency of irAEs appears to upsurge consequently (198). The microbiome has been related to the danger of creating immune-related noxiousness. The abundance of species according to study by Dubin et al. (202) among Bacteroidetes phylum, explicitly *Barnesiellaceae*, *Bacteroidaceae*, and *Rikenellaceae*, was related with resistance to colitis in patients with metastatic melanoma dealt with ipilimumab (n=34), though reduced recognition of hereditary pathways engaged with polyamine transport and vitamin B amalgamation in the gut related with an expanded danger of colitis (202).

Moreover, Chaput et al. showed that baseline gut microbiota is a good predictor of clinical response and colitis in metastatic melanoma patients treated with ipilimumab. Twenty-six patients with metastatic melanoma treated with ipilimumab were enrolled in the study. Fecal microbiota composition was assessed at baseline and before each ipilimumab infusion. In their results, baseline gut microbiota enriched with *Faecalibacterium* and other Firmicutes was associated with beneficial clinical response compared with patients whose baseline microbiota was driven by *Bacteroides* (194).

The significant reason for repeat and poor prognosis is the treatment failure in colorectal cancer patients, resistance to malignancy medicines has been connected to the nearness of explicit sorts of microbes in the gut. In colorectal cancer patients, scientists observed that resistance to drugs correlated with an expansion in *F. nucleatum* in the gut. The bacterium appeared to square passing (apoptosis) of the malignant growth cells and trigger autophagy, an endurance device for the disease cells. Yu et al. explored the commitment of gut microbiome to chemoresistance in patients with colorectal malignant growth (203). In colorectal cancer tissues in patients with relapse post-chemotherapy, the *F. nucleatum* was found in abundance and

TABLE 4 | Ongoing clinical trials of gut microbiota modulation in potentiating efficacy of anticancer therapies.

Patient /Cancer	Number of subjects	Intervention	Primary outcomes	Secondary outcome	Location	Status	Clinical trial registration number
Chemotherapy							
Patients with metastatic CRC	50	Chemotherapy + Weileshu (*Lactobacillus salivarius* AP-32, *Lactobacillus johnsonii* MH-68) vs chemotherapy alone	PFS	OS	Zhejiang, China	Not yet recruiting	NCT04021589
Patients with metastatic CRC	140	Chemotherapy + targeted therapy + Bifico (*Lactobacillus acidophilus* and *Bifidobacterium*) vs chemotherapy + targeted therapy	ORR	/	Zhejiang, China	Not yet recruiting	NCT04131803
Rectal cancer patients receiving concurrent chemotherapy and pelvic Radiation therapy	160	VSL#3 vs placebo	Impact of probiotics to increase tumor regression grade (TRG) 1-2 rate	Acute bowel toxicity Pathological complete response Sphincter saving surgery Disease-free survival Late toxicity (at 12-36 months)	Rome, Italy	Recruiting	NCT01579591
Immunotherapy							
Melanoma patients resistant/refractory to PD-1 therapy	20	Single-arm: FMT from anti-PD1 responders through colonoscopy + PD-1 therapy	ORR	T cell composition T cell function Immune profile	Pennsylvania, United States	Recruiting	NCT03341143
Patients with solid tumors (including non-small cell lung cancer, renal cell carcinoma, bladder cancer or melanoma	132	Single-arm: MRx0518 + Pembrolizumab	Adverse events	Tumor biomarkers Clinical benefits (ORR, DOR, DCR, PFS) Microbiome composition OS	Texas, United States	Recruiting	NCT03637803

PFS, progression-free survival; OS, overall survival; ORR, objective response rate; DOR, duration of response; DCR, disease control rate.

was related to patient clinicopathological qualities. They additionally showed that *F. nucleatum* elevated colorectal malignant growth protection from chemotherapy. *F. nucleatum*, mechanistically, targets TLR4 and MYD88 inborn immune signaling and specific microRNAs to enact the autophagy pathway and modify colorectal malignant growth chemotherapeutic reaction. To control colorectal cancer chemoresistance mechanistically, clinically, and biologically, the F. nucleatum arranges an atomic system of the Toll-like receptor, autophagy, and micro-RNAs. Estimating and focusing on *F. nucleatum* and its related pathway will yield critical understanding into clinical administration and may enhance colorectal malignant patient outcomes (203).

Albeit a progression of examinations has confirmed the effect of the microbiome on disease treatment, there still lies a lot of vagueness and deficiency in these investigations. The initial test experienced is the inadequate comprehension of the rare microbial species engaged with a better strong reaction.

The Potential of Microbiome in Cancer Therapy

The worldwide malignant growth trouble has risen drastically, making it a critical need to create novel treatments and anticipate which treatment will offer the most advantage to a disease quiet. The use of microorganisms to treat tumors is nothing new. German physician W Busch probably treated the first patient with cancer to be purposefully infected with bacteria in 1868 (204). He induced a bacterial infection in a woman with an inoperable sarcoma by first cauterizing the tumor and then placing her into bedding previously occupied by a patient with "erysipelas" (*Streptococcus pyogenes*). Busch reported that within a week, the primary tumor and the lymph nodes in the neck had shrunk in size. Unfortunately, the patient died a few days after the infection had begun. Later, in 1883 Friedrich Fehleisen, a German surgeon had identified *S. pyogenes* as the cause of "erysipelas" and had begun treating patients with cancer with the living cultures of the bacteria with success (205). Almost simultaneously in the USA, William B. Coley was performing similar experiments. In 1981 he injected *Streptococcus* into a patient with inoperable cancer (206). The infection caused by the bacterium has the side effect of shrinking the malignant tumor, and this was one of the first examples of immunotherapy. Given the risks of using live organisms, Coley developed a mixture of dead bacteria to treat his patients. They were known as Coley's toxins. He then devoted most of his life as head of the Bone Tumor Service at Memorial Hospital in New York, treating more than 1000 cancer patients with bacteria or bacterial products (207). However, his studies, the first using immunotherapy, were forgotten for several reasons. First, the use of microorganism(s) to treat human cancers provided a short term benefit, but eventually, tumors recurred; second, the discovery of radioactivity and its use to treat tumors in the early 1930s and second the advent of chemotherapy in the 1940s relegated the use of immunotherapies on a second plane.

In recent years, there has been a renewed interest in using bacteria-mediated tumor therapy (204, 208, 209). Although widely used for treating many tumors, chemotherapy causes many off-target effects, such as significant damage to healthy tissues. In contrast, biological generally exert target-specific effects and are relatively safer for human use. Moreover, bacterial-based immunotherapies can penetrate solid tumors and inexpensive. Upon systemic administration, various types of non-pathogenic obligate anaerobes and facultative anaerobes have been shown to infiltrate and selectively replicate within solid tumors (204, 209).

A significant step in the development of bacterial therapeutics is identifying potential species and strains with minimal pathogenicity to the host, and that can replicate precisely in the tumor hypoxic microenvironment. Several genera of bacteria, including *Clostridium*, *Bifidobacterium*, *E. coli*, and attenuated *Salmonella*, have been used in bacteria-mediated tumor therapy (204, 208–210).

The genera *Clostridium* and *Salmonella* are probable the best-studied as vectors in bacteria-mediated tumor therapy. In 1947, it was first shown that direct injection of spores of *Clostridium histolyticus* into a transplantable mouse sarcoma caused oncolysis and tumor regression; however, mice died soon after (211). In 1964 Moese and Moese injected a non-pathogenic isolate of *Clostridium butyricum* intravenously and observed the tumor's disappearance, but again the survival of the mice was non-permanent (212). The ability of *Clostridium* to grow in hypoxic areas with necrosis gives them an advantage to target tumors. With the development of genetic systems to work with *Clostridium*, this bacterium has attracted renewed interest as a potential vector to treat solid tumors. Thus, a genetically engineered *C. acetobutylicum* expressing and secreting the *E. coli* cytosine deaminase (CDase) has been used against rhabdomyosarcoma-bearing WAG/Rij rats and show that the enzyme was produced in tumors *in vivo* (213).

Numerous *S. typhimurium* mutant strains have been studied from the perspective of cancer treatment. One of the significant advantages of using *S. typhimurium* is that there is a well-developed genetic system that allows for all kinds of genetic manipulations of this organism. Many different immunomodulators have been cloned into *S. typhimurium* to be expressed at the tumor site. These include cytotoxic proteins, to kill tumor cells directly, and tumor-associated antigens to increase the immune response in the site of the tumor, and prodrug enzymes that modify a substrate to convert it into a toxic product (214, 215).

So far, the only strain of *S. typhimurium* going through a phase I clinical trial is strain VNP20009, which contains deletions in the *msb*B and *pur*l genes, to attenuate virulence and avoid septic shock (216).

A more recent approach has been combining bacteria-mediated cancer therapies with other kinds of therapies. For example, a photo-thermal agent, such as melanin-like poly-dopamine (pDA), was coated with VNP20009 targeted to hypoxic and necrotic tumor areas. A mouse model of the tumor was irradiated with a near-infrared laser, which achieved tumor targeting and tumor elimination without relapse or metastasis (217).

Although no reports on the treatment of OSCC have been published, the potential of these techniques to treat oral tumors is enormous. OSCC are solid tumors, and they are easily accessible to be treated at the site, which makes the ideal type of cancer treated with bacterial-mediated tumor therapies.

THE FUTURE OF MICROBIOME IN CANCER THERAPY

We anticipate that the human microbiome will bit by bit play an undeniably conspicuous role in cancer treatment. As of now, the system of the microbiome's impacts in cancer treatment is not surely known; be that as it may, some clinical pilot studies will assist with uncovering the capability of the microbiome in tumor advancement and malignancy treatment. The advancement of these clinical preliminaries will expel impediments for utilizing the microbiome to improve and help treatment utilizing ICIs. The microbiome, at first, can lessen complexities during malignant growth treatment. Right now, the most widely recognized noxious reaction when utilizing ICIs is related to colitis. The reason for the ailment is uncertain. Curiously, the lactic acid bacterium *Lactobacillus reuteri* can wipe out ICIs related to colitis and improve weight reduction and irritation (218). Second, the intestinal microbiome upgrades the nourishing assimilation limit of patients with cancer and improves their anti-tumor capacity. The rise of tumor micro-ecological immune nutrition has additionally prepared for the improvement of the microbiome as implement in disease immunotherapy. Third, microbiome research is relied upon to prompt the structure of immunization against tumors. An ongoing microbial-based malignancy immunization has demonstrated its utility. This malignant growth antibody forestalls the development of squamous cell carcinoma communicating epidermal growth factor receptor (EGFR) vIII and instigates EGFR vIII-explicit cell insusceptibility (219).

Current research is thrilling for investigation against tumor resistance and signifies a discovery in the structure of the microbiome. For cancer immunotherapy, FMT is predictable to be the most critical immediate bio optimization tool. FMT is a mainstream innovation that has been utilized clinically to treat repetitive *Clostridium difficile* contaminations (220).

Finally, recent studies support the hypothesis that periodontal inflammation exacerbates gut inflammation *in vivo* by translocation of oral pathobionts to the gut, activating the inflammasome in colonic mononuclear phagocytes resulting in inflammation (221, 222). Additionally, periodontitis results in the generation of reactive Th17 cells against oral bacteria. These reactive Th17 cells are exhibit gut tropism and migrate to the inflamed gut. When in the gut, Th17 cells of oral origin can be activated by translocated oral pathobionts and cause the development of colitis, but gut-resident microbes do not activate them. We do not know if gut inflammation could have a similar effect on the oral cavity's inflammatory environment, as a result of which the severity of head and neck cancer could increase. Studies of the gut microbiome's effect on the immune response to head and neck cancer are lacking but maybe worth pursuing.

AUTHOR CONTRIBUTIONS

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REFERENCES

1. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol* (2016) 14:1–14. doi: 10.1371/journal.pbio.1002533
2. Group TNHW, Peterson J, Garges S, Giovanni M, McInnes P, Wang L, et al. The NIH Human Microbiome Project. *Genome Res* (2009) 19:2317–23. doi: 10.1101/gr.096651.109
3. Fukasawa A, Kurita-Ochiai T, Hashizume T, Kobayashi R, Akimoto Y, Yamamoto M. Porphyromonas gingivalis accelerates atherosclerosis in C57BL/6 mice fed a high-fat diet. *Immunopharmacol Immunotoxicol* (2012) 34:470–6. doi: 10.3109/08923973.2011.627866
4. Gavin PG, Mullaney JA, Loo D, Cao K-AL, Gottlieb PA, Hill MM, et al. Intestinal Metaproteomics Reveals Host-Microbiota Interactions in Subjects at Risk for Type 1 Diabetes. *Diabetes Care* (2018) 41:2178–86. doi: 10.2337/dc18-0777
5. Ling Z, Li Z, Liu X, Cheng Y, Luo Y, Tong X, et al. Altered Fecal Microbiota Composition Associated with Food Allergy in Infants. *Appl Environ Microbiol* (2014) 80:2546–54. doi: 10.1128/AEM.00003-14
6. Sartor RB, Mazmanian SK. Intestinal Microbes in Inflammatory Bowel Diseases. *Am J Gastroenterol Suppl* (2012) 1:15–21. doi: 10.1038/ajgsup.2012.4
7. Tamboli CP, Neut C, Desreumaux P, Colombel JF. Dysbiosis in inflammatory bowel disease. *Gut* (2004) 53:1–4. doi: 10.1136/gut.53.1.1
8. de Martel C, Georges D, Bray F, Ferlay J, Clifford GM. Global burden of cancer attributable to infections in 2018: a worldwide incidence analysis. *Lancet Glob Health* (2020) 8:e180–90. doi: 10.1016/S2214-109X(19)30488-7
9. Moore PS, Chang YH. Why do viruses cause cancer? Highlights of the first century of human tumour virology. *Nat Rev Cancer* (2010) 10:878–89. doi: 10.1038/nrc2961
10. Marshall Barry J, Warren JR, Blincow Elizabeth D, Phillips M, Goodwin CS, Murray R, et al. Prospective Double-Blind Trial of Duodenal Ulcer Relapse After Eradication of Campylobacter Pylori. *Lancet* (1988) 332:1437–42. doi: 10.1016/S0140-6736(88)90929-4
11. Amieva M, Peek RM. Pathobiology of Helicobacter pylori-induced Gastric Cancer. *Gastroenterology* (2015) 150:64–78. doi: 10.1053/j.gastro.2015.09.004
12. Parsonnet J. Helicobacter pylori and gastric cancer. *Gastroenterol Clin North Am* (1993) 22:89–104.
13. Wang F, Meng W, Wang B, Qiao L. Helicobacter pylori-induced gastric inflammation and gastric cancer. *Cancer Lett* (2014) 345:196–202. doi: 10.1016/j.canlet.2013.08.016
14. Fox JG, Wang TC. Inflammation, atrophy, and gastric cancer. *J Clin Invest* (2007) 117:60–9. doi: 10.1172/JCI30111
15. Virchow R, Virchow R. *Die krankhaften Geschwülste: dreissig Vorlesungen, gehalten während des Wintersemesters 1862-1863 an der Universität zu Berlin*. Berlin: Verlag von August Hirschwald (1863).
16. Bhatelia K, Singh K, Singh R. TLRs: linking inflammation and breast cancer. *Cell Signal* (2014) 26:2350–7. doi: 10.1016/j.cellsig.2014.07.035

17. Cutolo M, Paolino S, Pizzorni C. Possible contribution of chronic inflammation in the induction of cancer in rheumatic diseases. *Clin Exp Rheumatol* (2014) 32:839–47.
18. Wang D, DuBois RN. Immunosuppression associated with chronic inflammation in the tumor microenvironment. *Carcinogenesis* (2015) 36:1085–93. doi: 10.1093/carcin/bgv123
19. Kipanyula MJ, Seke Etet PF, Vecchio L, Farahna M, Nukenine EN, Nwabo Kamdje AH. Signaling pathways bridging microbial-triggered inflammation and cancer. *Cell Signal* (2013) 25:403–16. doi: 10.1016/j.cellsig.2012.10.014
20. Chang AH, Parsonnet J. Role of Bacteria in Oncogenesis. *Clin Microbiol Rev* (2010) 23:837–57. doi: 10.1128/CMR.00012-10
21. Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, et al. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Res* (2012) 22:292–8. doi: 10.1101/gr.126573.111
22. Whitmore SE, Lamont RJ. Oral Bacteria and Cancer. *PLoS Pathog* (2014) 10: e1003933. doi: 10.1371/journal.ppat.1003933
23. Schwabe RF, Jobin C. The microbiome and cancer. *Nat Rev Cancer* (2013) 13:800–12. doi: 10.1038/nrc3610
24. Arthur JC, Gharaibeh RZ, Mühlbauer M, Perez-Chanona E, Uronis JM, McCafferty J, et al. Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer. *Nat Commun* (2014) 5:4724. doi: 10.1038/ncomms5724
25. Ohadian Moghadam S, Momeni SA. Human microbiome and prostate cancer development: current insights into the prevention and treatment. *Front Med* (2020) 14(3):1–22. doi: 10.1007/s11684-019-0731-7
26. Dutta U, Garg PK, Kumar R, Tandon RK. Typhoid carriers among patients with gallstones are at increased risk for carcinoma of the gallbladder. *Am J Gastroenterol* (2000) 95:784–7. doi: 10.1111/j.1572-0241.2000.01860.x
27. Lax AJ, Thomas W. How bacteria could cause cancer: one step at a time. *Trends Microbiol* (2002) 10:293–9. doi: 10.1016/s0966-842x(02)02360-0
28. Vaishnavi C, Kochhar R, Singh G, Kumar S, Singh S, Singh K. Epidemiology of typhoid carriers among blood donors and patients with biliary, gastrointestinal and other related diseases. *Microbiol Immunol* (2005) 49:107–12. doi: 10.1111/j.1348-0421.2005.tb03709.x
29. Ellmerich S, Schöller M, Duranton B, Gossé F, Galluser M, Klein JP, et al. Promotion of intestinal carcinogenesis by Streptococcus bovis. *Carcinogenesis* (2000) 21:753–6. doi: 10.1093/carcin/21.4.753
30. Gold JS, Bayar S, Salem RR. Association of Streptococcus bovis bacteremia with colonic neoplasia and extracolonic malignancy. *Arch Surg* (2004) 139:760–5. doi: 10.1001/archsurg.139.7.760
31. Zarkin BA, Lillemoe KD, Cameron JL, Effron PN, Magnuson TH, Pitt HA. The triad of Streptococcus bovis bacteremia, colonic pathology, and liver disease. *Ann Surg* (1990) 211:786–791; discussion 791-792. doi: 10.1097/00000658-199006000-00019
32. Anttila T, Koskela P, Leinonen M, Laukkanen P, Hakulinen T, Lehtinen M, et al. Chlamydia pneumoniae infection and the risk of female early-onset lung cancer. *Int J Cancer* (2003) 107:681–2. doi: 10.1002/ijc.11353
33. Hua-Feng X, Yue-Ming W, Hong L, Junyi D. A meta-analysis of the association between Chlamydia pneumoniae infection and lung cancer risk. *Indian J Cancer* (2015) 52(Suppl 2):e112–115. doi: 10.4103/0019-509X.172506
34. Khan S, Imran A, Khan AA, Abul Kalam M, Alshamsan A. Systems Biology Approaches for the Prediction of Possible Role of Chlamydia pneumoniae Proteins in the Etiology of Lung Cancer. *PLoS One* (2016) 11:e0148530. doi: 10.1371/journal.pone.0148530
35. Koyi H, Brandén E, Gnarpe J, Gnarpe H, Steen B. An association between chronic infection with Chlamydia pneumoniae and lung cancer. A prospective 2-year study. *APMIS* (2001) 109:572–80. doi: 10.1034/j.1600-0463.2001.d01-177.x
36. Littman AJ, White E, Jackson LA, Thornquist MD, Gaydos CA, Goodman GE, et al. Chlamydia pneumoniae infection and risk of lung cancer. *Cancer Epidemiol Biomarkers Prev* (2004) 13:1624–30. doi: 10.1016/S1047-2797(03)00148-0
37. Boleij A, van Gelder MMHJ, Swinkels DW, Tjalsma H. Clinical Importance of Streptococcus gallolyticus infection among colorectal cancer patients: systematic review and meta-analysis. *Clin Infect Dis* (2011) 53:870–8. doi: 10.1093/cid/cir609
38. Bronzato JD, Bomfim RA, Edwards DH, Crouch D, Hector MP, Gomes BPFA. Detection of Fusobacterium in oral and head and neck cancer samples: A systematic review and meta-analysis. *Arch Oral Biol* (2020) 112:104669. doi: 10.1016/j.archoralbio.2020.104669
39. Krishnan S, Eslick GD. Streptococcus bovis infection and colorectal neoplasia: a meta-analysis. *Colorectal Dis* (2014) 16:672–80. doi: 10.1111/codi.12662
40. Peng B-J, Cao C-Y, Li W, Zhou Y-J, Zhang Y, Nie Y-Q, et al. Diagnostic Performance of Intestinal Fusobacterium nucleatum in Colorectal Cancer: A Meta-Analysis. *Chin Med J* (2018) 131:1349–56. doi: 10.4103/0366-6999.232814
41. Repass JReproducibility Project: Cancer Biology, , Iorns E, Denis A, Williams SR, Perfito N, et al. Replication Study: Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Elife* (2018) 7:1–12. doi: 10.7554/eLife.25801
42. Zhan P, Suo L, Qian Q, Shen X, Qiu L-X, Yu L, et al. Chlamydia pneumoniae infection and lung cancer risk: a meta-analysis. *Eur J Cancer* (2011) 47:742–7. doi: 10.1016/j.ejca.2010.11.003
43. Hundal R, Shaffer EA. Gallbladder cancer: epidemiology and outcome. *Clin Epidemiol* (2014) 6:99–109. doi: 10.2147/CLEP.S37357
44. Welton JC, Marr JS, Friedman SM. Association between hepatobiliary cancer and typhoid carrier state. *Lancet* (1979) 1:791–4. doi: 10.1016/s0140-6736(79)91315-1
45. Koshiol J, Wozniak A, Cook P, Adaniel C, Acevedo J, Azócar L, et al. Salmonella enterica serovar Typhi and gallbladder cancer: a case-control study and meta-analysis. *Cancer Med* (2016) 5:3310–235. doi: 10.1002/cam4.915
46. Dutta U, Bush N, Kalsi D, Popli P, Kapoor VK. Epidemiology of gallbladder cancer in India. *Chin Clin Oncol* (2019) 8:33. doi: 10.21037/cco.2019.08.03
47. Nagaraja V, Eslick GD. Systematic review with meta-analysis: the relationship between chronic Salmonella typhi carrier status and gallbladder cancer. *Aliment Pharmacol Ther* (2014) 39:745–50. doi: 10.1111/apt.12655
48. Scanu T, Spaapen RM, Bakker JM, Pratap CB, Wu L, Hofland I, et al. Salmonella Manipulation of Host Signaling Pathways Provokes Cellular Transformation Associated with Gallbladder Carcinoma. *Cell Host Microbe* (2015) 17:763–74. doi: 10.1016/j.chom.2015.05.002
49. Couturier-Maillard A, Secher T, Rehman A, Normand S, De Arcangelis A, Haesler R, et al. NOD2-mediated dysbiosis predisposes mice to transmissible colitis and colorectal cancer. *J Clin Invest* (2013) 123:700–11. doi: 10.1172/JCI62236
50. Zackular JP, Baxter NT, Iverson KD, Sadler WD, Petrosino JF, Chen GY, et al. The gut microbiome modulates colon tumorigenesis. *MBio* (2013) 4: e00692–00613. doi: 10.1128/mBio.00692-13
51. Sheflin AM, Whitney AK, Weir TL. Cancer-Promoting Effects of Microbial Dysbiosis. *Curr Oncol Rep* (2014) 16:406. doi: 10.1007/s11912-014-0406-0
52. La Rosa GRM, Gattuso G, Pedullà E, Rapisarda E, Nicolosi D, Salmeri M. Association of oral dysbiosis with oral cancer development. *Oncol Lett* (2020) 19:3045–58. doi: 10.3892/ol.2020.11441
53. Cook TJ. Blood dyscrasias as related to periodontal disease; with special reference to leukemia. *J Periodontol* (1947) 18:159–65. doi: 10.1902/jop.1947.18.4.159
54. Wentz FM. Oral Manifestations of the Blood Diseases. *J Am Dental Assoc* (1952) 44:698–701. doi: 10.14219/jada.archive.1952.0114
55. Corbella S, Veronesi P, Galimberti V, Weinstein R, Del Fabbro M, Francetti L. Is periodontitis a risk indicator for cancer? A meta-analysis. *PLoS One* (2018) 13:e0195683. doi: 10.1371/journal.pone.0195683
56. Wu Y, Shi X, Li Y, Shi X, Gu Y, Qian Q, et al. Hematopoietic and lymphatic cancers in patients with periodontitis: a systematic review and meta-analysis. *Med Oral Patol Oral Cir Bucal* (2020) 25:e21–8. doi: 10.4317/medoral.23166
57. Bracci PM. Oral Health and the Oral Microbiome in Pancreatic Cancer: An Overview of Epidemiological Studies. *Cancer J* (2017) 23:310–4. doi: 10.1097/PPO.0000000000000287
58. Maisonneuve P, Amar S, Lowenfels AB. Periodontal disease, edentulism, and pancreatic cancer: a meta-analysis. *Ann Oncol* (2017) 28:985–95. doi: 10.1093/annonc/mdx019
59. Michaud DS, Fu Z, Shi J, Chung M. Periodontal Disease, Tooth Loss, and Cancer Risk. *Epidemiol Rev* (2017) 39:49–58. doi: 10.1093/epirev/mxx006

60. Xiao L, Zhang Q, Peng Y, Wang D, Liu Y. The effect of periodontal bacteria infection on incidence and prognosis of cancer: A systematic review and meta-analysis. *Medicine* (2020) 99:e19698. doi: 10.1097/MD.0000000000019698
61. Shi T, Min M, Sun C, Zhang Y, Liang M, Sun Y. Periodontal disease and susceptibility to breast cancer: A meta-analysis of observational studies. *J Clin Periodontol* (2018) 45:1025–33. doi: 10.1111/jcpe.12982
62. Ren HG, Luu HN, Cai H, Xiang YB, Steinwandel M, Gao YT, et al. Oral health and risk of colorectal cancer: results from three cohort studies and a meta-analysis. *Ann Oncol* (2016) 27:1329–36. doi: 10.1093/annonc/mdw172
63. Ochi A, Nguyen AH, Bedrosian AS, Mushlin HM, Zarbakhsh S, Barilla R, et al. MyD88 inhibition amplifies dendritic cell capacity to promote pancreatic carcinogenesis via Th2 cells. *J Exp Med* (2012) 209:1671–87. doi: 10.1084/jem.20111706
64. Peters BA, Wu J, Pei Z, Yang L, Purdue MP, Freedman ND, et al. Oral microbiome composition reflects prospective risk for esophageal cancers. *Cancer Res* (2017) 77(23):6777–87.
65. Narikiyo M, Tanabe C, Yamada Y, Igaki H, Tachimori Y, Kato H, et al. Frequent and preferential infection of Treponema denticola, Streptococcus mitis, and Streptococcus anginosus in esophageal cancers. *Cancer Sci* (2004) 95(7):569–74.
66. Snider EJ, Compres G, Freedberg DE, Giddins MJ, Khiabanian H, Lightdale CJ, et al. Barrett's esophagus is associated with a distinct oral microbiome. *Clin Transl Gastroenterol* (2018) 9:135. doi: 10.1038/s41424-018-0005-8
67. Chen X, Winckler B, Lu M, Cheng H, Yuan Z, Yang Y, et al. Oral Microbiota and Risk for Esophageal Squamous Cell Carcinoma in a High-Risk Area of China. *PLoS One* (2015) 10:e0143603. doi: 10.1371/journal.pone.0143603
68. Fan X, Alekseyenko AV, Wu J, Peters BA, Jacobs EJ, Gapstur SM, et al. Human oral microbiome and prospective risk for pancreatic cancer: a population-based nested case-control study. *Gut* (2018) 67:120–7. doi: 10.1136/gutjnl-2016-312580
69. Michaud DS, Izard J, Wilhelm-Benartzi CS, You D-H, Grote VA, Tjønneland A, et al. Plasma antibodies to oral bacteria and risk of pancreatic cancer in a large European prospective cohort study. *Gut* (2013) 62:1764–70. doi: 10.1136/gutjnl-2012-303006
70. Mitsuhashi K, Nosho K, Sukawa Y, Matsunaga Y, Ito M, Kurihara H, et al. Association of Fusobacterium species in pancreatic cancer tissues with molecular features and prognosis. *Oncotarget* (2015) 6:7209–20. doi: 10.18632/oncotarget.3109
71. Farrell JJ, Zhang L, Zhou H, Chia D, Elashoff D, Akin D, et al. Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer. *Gut* (2012) 61:582–8. doi: 10.1136/gutjnl-2011-300784
72. Torres PJ, Fletcher EM, Gibbons SM, Bouvet M, Doran KS, Kelley ST. Characterization of the salivary microbiome in patients with pancreatic cancer. *PeerJ* (2015) 3:e1373. doi: 10.7717/peerj.1373
73. Vogtmann E, Han Y, Caporaso JG, Bokulich N, Mohamadkhani A, Moayyedkazemi A, et al. Oral microbial community composition is associated with pancreatic cancer: A case-control study in Iran. *Cancer Med* (2020) 9:797–806. doi: 10.1002/cam4.2660
74. Del Castillo E, Meier R, Chung M, Koestler DC, Chen T, Paster BJ, et al. The Microbiomes of Pancreatic and Duodenum Tissue Overlap and Are Highly Subject Specific but Differ between Pancreatic Cancer and Noncancer Subjects. *Cancer Epidemiol Biomarkers Prev* (2019) 28:370–83. doi: 10.1158/1055-9965.EPI-18-0542
75. Olson SH, Satagopan J, Xu Y, Ling L, Leong S, Orlow I, et al. The oral microbiota in patients with pancreatic cancer, patients with IPMNs, and controls: a pilot study. *Cancer Causes Control* (2017) 28:959–69. doi: 10.1007/s10552-017-0933-8
76. Lu H, Ren Z, Li A, Li J, Xu S, Zhang H, et al. Tongue coating microbiome data distinguish patients with pancreatic head cancer from healthy controls. *J Oral Microbiol* (2019) 11:1563409. doi: 10.1080/20002297.2018.1563409
77. Lu H, Ren Z, Li A, Zhang H, Jiang J, Xu S, et al. Deep sequencing reveals microbiota dysbiosis of tongue coat in patients with liver carcinoma. *Sci Rep* (2016) 6:33142.
78. Yan X, Yang M, Liu J, Gao R, Hu J, Li J, et al. Discovery and validation of potential bacterial biomarkers for lung cancer. *Am J Cancer Res* (2015) 5 (10):3111.
79. Tsay J-CJ, Wu BG, Badri MH, Clemente JC, Shen N, Meyn P, et al. Airway microbiota is associated with upregulation of the PI3K pathway in lung cancer. *Am J respiratory Crit Care Med* (2018) 198 (9):1188–98.
80. Yang C-Y, Yeh Y-M, Yu H-Y, Chin C-Y, Hsu C-W, Liu H, et al. Oral Microbiota Community Dynamics Associated With Oral Squamous Cell Carcinoma Staging. *Front Microbiol* (2018) 9:862. doi: 10.3389/fmicb.2018.00862
81. Zhang W, Luo J, Dong X, Zhao S, Hao Y, Peng C, et al. Salivary microbial dysbiosis is associated with systemic inflammatory markers and predicted oral metabolites in non-small cell lung cancer patients. *J Cancer* (2019) 10 (7):1651.
82. Yang Y, Zheng W, Cai Q-Y, Shrubsole MJ, Pei Z, Brucker R, et al. Cigarette smoking and oral microbiota in low-income and African-American populations. *J Epidemiol Community Health* (2019) 73(12):1108–15.
83. Ahn J, Sinha R, Pei Z, Dominianni C, Wu J, Shi J, et al. Human gut microbiome and risk for colorectal cancer. *J Natl Cancer Institute* (2013) 105 (24):1907–11.
84. Tahara T, Yamamoto E, Suzuki H, Maruyama R, Chung W, Garriga J, et al. Fusobacterium in colonic flora and molecular features of colorectal carcinoma. *Cancer Res* (2014) 74:1311–8. doi: 10.1158/0008-5472.CAN-13-1865
85. Kato I, Vasquez AA, Moyerbrailean G, Land S, Sun J, Lin H-S, et al. Oral microbiome and history of smoking and colorectal cancer. *J epidemiological Res* (2016) 2(2):92.
86. Flemer B, Warren RD, Barrett MP, Cisek K, Das A, Jeffery IB, et al. The oral microbiota in colorectal cancer is distinctive and predictive. *Gut* (2018) 67:1454–63. doi: 10.1136/gutjnl-2017-314814
87. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* (2013) 14:207–15. doi: 10.1016/j.chom.2013.07.007
88. Flanagan L, Schmid J, Ebert M, Soucek P, Kunicka T, Liska V, et al. Fusobacterium nucleatum associates with stages of colorectal neoplasia development, colorectal cancer and disease outcome. *Eur J Clin Microbiol Infect Dis* (2014) 33:1381–90. doi: 10.1007/s10096-014-2081-3
89. Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, et al. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res* (2012) 22:299–306. doi: 10.1101/gr.126516.111
90. Gao Z, Guo B, Gao R, Zhu Q, Qin H. Microbiota disbiosis is associated with colorectal cancer. *Front Microbiol* (2015) 6:20:1–9. doi: 10.3389/fmicb.2015.00020
91. Kageyama S, Takeshita T, Takeuchi K, Asakawa M, Matsumi R, Furuta M, et al. Characteristics of the Salivary Microbiota in Patients With Various Digestive Tract Cancers. *Front Microbiol* (2019) 10:1780. doi: 10.3389/fmicb.2019.01780
92. Drewes JL, White JR, Dejea CM, Fathi P, Iyadorai T, Vadivelu J, et al. High-resolution bacterial 16S rRNA gene profile meta-analysis and biofilm status reveal common colorectal cancer consortia. *NPJ Biofilms Microbiomes* (2017) 3:34. doi: 10.1038/s41522-017-0040-3
93. Thomas AM, Manghi P, Asnicar F, Pasolli E, Armanini F, Zolfo M, et al. Metagenomic analysis of colorectal cancer datasets identifies cross-cohort microbial diagnostic signatures and a link with choline degradation. *Nat Med* (2019) 25:667–78. doi: 10.1038/s41591-019-0405-7
94. Hu J, Han S, Chen Y, Ji Z. Variations of tongue coating microbiota in patients with gastric cancer. *BioMed Res Int* (2015) 2015.
95. Sun J-H, Li X-L, Yin J, Li Y-H, Hou B-X, Zhang Z. A screening method for gastric cancer by oral microbiome detection. *Oncol Rep* (2018) 39:2217–24. doi: 10.3892/or.2018.6286
96. Wu J, Xu S, Xiang C, Cao Q, Li Q, Huang J, et al. Tongue Coating Microbiota Community and Risk Effect on Gastric Cancer. *J Cancer* (2018) 9:4039–48. doi: 10.7150/jca.25280
97. Wang H, Altemus J, Niazi F, Green H, Calhoun BC, Sturgis C, et al. Breast tissue, oral and urinary microbiomes in breast cancer. *Oncotarget* (2017) 8 (50):88122.
98. Hieken TJ, Chen J, Hoskin TL, Walther-Antonio M, Johnson S, Ramaker S, et al. The microbiome of aseptically collected human breast tissue in benign and malignant disease. *Sci Rep* (2016) 6:30751.

99. Byrd V, Getz T, Padmanabhan R, Arora H, Eng C. The microbiome in PTEN hamartoma tumor syndrome. *Endocrine-related Cancer* (2018) 25(3):233–43.
100. Shang F-M, Liu H-L. Fusobacterium nucleatum and colorectal cancer: A review. *World J Gastrointest Oncol* (2018) 10:71–81. doi: 10.4251/wjgo.v10.i3.71
101. Cook NR. Methods for Evaluating Novel Biomarkers - a New Paradigm. *Int J Clin Pract* (2010) 64:1723–7. doi: 10.1111/j.1742-1241.2010.02469.x
102. Zheng D-W, Dong X, Pan P, Chen K-W, Fan J-X, Cheng S-X, et al. Phage-guided modulation of the gut microbiota of mouse models of colorectal cancer augments their responses to chemotherapy. *Nat BioMed Eng* (2019) 3:717–28. doi: 10.1038/s41551-019-0423-2
103. Komiya Y, Shimomura Y, Higurashi T, Sugi Y, Arimoto J, Umezawa S, et al. Patients with colorectal cancer have identical strains of Fusobacterium nucleatum in their colorectal cancer and oral cavity. *Gut* (2019) 68:1335–7. doi: 10.1136/gutjnl-2018-316661
104. Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin. *Cell Host Microbe* (2013) 14:195–206. doi: 10.1016/j.chom.2013.07.012
105. Han YW. Fusobacterium nucleatum: a commensal-turned pathogen. *Curr Opin Microbiol* (2015) 23C:141–7. doi: 10.1016/j.mib.2014.11.013
106. Chen Y, Peng Y, Yu J, Chen T, Wu Y, Shi L, et al. Invasive Fusobacterium nucleatum activates beta-catenin signaling in colorectal cancer via a TLR4/P-PAK1 cascade. *Oncotarget* (2017) 8:31802–14. doi: 10.18632/oncotarget.15992
107. Nelson WJ, Nusse R. Convergence of Wnt, β-Catenin, and Cadherin Pathways. *Science* (2004) 303:1483–7. doi: 10.1126/science.1094291
108. Tian X, Liu Z, Niu B, Zhang J, Tan TK, Lee SR, et al. E-Cadherin/β-Catenin Complex and the Epithelial Barrier. *J BioMed Biotechnol* (2011) 2011:1–7. doi: 10.1155/2011/567305
109. Rubinstein MR, Baik JE, Lagana SM, Han RP, Raab WJ, Sahoo D, et al. Fusobacterium nucleatum promotes colorectal cancer by inducing Wnt/β-catenin modulator Annexin A1. *EMBO Rep* (2019) 20:1–17. doi: 10.15252/embr.201847638
110. Alao JP. The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Mol Cancer* (2007) 6:24. doi: 10.1186/1476-4598-6-24
111. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* (2018) 68:394–424. doi: 10.3322/caac.21492
112. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA: A Cancer J Clinicians* (2020) 70:7–30. doi: 10.3322/caac.21590
113. Huber MA, Tantiwongkosi B. Oral and oropharyngeal cancer. *Med Clin North Am* (2014) 98:1299–321. doi: 10.1016/j.mcna.2014.08.005
114. Mücke T, Wagenpfeil S, Kesting MR, Hölzle F, Wolff K-D. Recurrence interval affects survival after local relapse of oral cancer. *Oral Oncol* (2009) 45:687–91. doi: 10.1016/j.oraloncology.2008.10.011
115. Rogers SN, Brown JS, Woolgar JA, Lowe D, Magennis P, Shaw RJ, et al. Survival following primary surgery for oral cancer. *Oral Oncol* (2009) 45:201–11. doi: 10.1016/j.oraloncology.2008.05.008
116. Wang B, Zhang S, Yue K, Wang X-D. The recurrence and survival of oral squamous cell carcinoma: a report of 275 cases. *Chin J Cancer* (2013) 32:614–8. doi: 10.5732/cjc.012.10219
117. Boysen M, Lövdal O, Tausjö J, Winther F. The value of follow-up in patients treated for squamous cell carcinoma of the head and neck. *Eur J Cancer* (1992) 28:426–30. doi: 10.1016/S0959-8049(05)80068-1
118. Braakhuis BJM, Tabor MP, Leemans CR, van der Waal I, Snow GB, Brakenhoff RH. Second primary tumors and field cancerization in oral and oropharyngeal cancer: molecular techniques provide new insights and definitions. *Head Neck* (2002) 24:198–206. doi: 10.1002/hed.10042
119. Schwartz GJ, Mehta RH, Wenig BL, Shaligram C, Portugal LG. Salvage treatment for recurrent squamous cell carcinoma of the oral cavity. *Head Neck* (2000) 22:34–41. doi: 10.1002/(SICI)1097-0347(200001)22:1<34::AID-HED6>3.0.CO;2-3
120. Liu S-A, Wong Y-K, Lin J-C, Poon C-K, Tung K-C, Tsai W-C. Impact of recurrence interval on survival of oral cavity squamous cell carcinoma patients after local relapse. *Otolaryngol Head Neck Surg* (2007) 136:112–8. doi: 10.1016/j.otohns.2006.07.002
121. Hardefeldt HA, Cox MR, Eslick GD. Association between human papillomavirus (HPV) and oesophageal squamous cell carcinoma: a meta-analysis. *Epidemiol Infect* (2014) 142:1119–37. doi: 10.1017/S0950268814000016
122. Liyanage SS, Rahman B, Ridda I, Newall AT, Tabrizi SN, Garland SM, et al. The aetiological role of human papillomavirus in oesophageal squamous cell carcinoma: a meta-analysis. *PLoS One* (2013) 8:e69238. doi: 10.1371/journal.pone.0069238
123. Petrick JL, Wyss AB, Butler AM, Cummings C, Sun X, Poole C, et al. Prevalence of human papillomavirus among oesophageal squamous cell carcinoma cases: systematic review and meta-analysis. *Br J Cancer* (2014) 110:2369–77. doi: 10.1038/bjc.2014.96
124. Syrjänen K, Syrjänen S. Detection of human papillomavirus in esophageal papillomas: systematic review and meta-analysis. *APMIS* (2013) 121:363–74. doi: 10.1111/apm.12003
125. Carpén T, Syrjänen S, Jouhi L, Randen-Brady R, Haglund C, Mäkitie A, et al. Epstein-Barr virus (EBV) and polyomaviruses are detectable in oropharyngeal cancer and EBV may have prognostic impact. *Cancer Immunol Immunother* (2020) 69:1615–26. doi: 10.1007/s00262-020-02570-3
126. Drop B, Strycharz-Dudziak M, Kliszczewska E, Polz-Dacewicz M. Coinfection with Epstein-Barr Virus (EBV), Human Papilloma Virus (HPV) and Polyoma BK Virus (BKPyV) in Laryngeal, Oropharyngeal and Oral Cavity Cancer. *Int J Mol Sci* (2017) 18: 1–13. doi: 10.3390/ijms18122752
127. Jalouli J, Jalouli MM, Sapkota D, Ibrahim SO, Larsson P-A, Sand L. Human papilloma virus, herpes simplex virus and epstein barr virus in oral squamous cell carcinoma from eight different countries. *Anticancer Res* (2012) 32:571–80.
128. Guerrero-Preston R, Godoy-Vitorino F, Jedlicka A, Rodríguez-Hilario A, González H, Bondy J, et al. 16S rRNA amplicon sequencing identifies microbiota associated with oral cancer, human papilloma virus infection and surgical treatment. *Oncotarget* (2016) 7(32):51320.
129. Tateda M, Shiga K, Saijo S, Sone M, Hori T, Yokoyama J, et al. Streptococcus anginosus in head and neck squamous cell carcinoma: implication in carcinogenesis. *Int J Mol Med* (2000) 6(6):699–1402.
130. Sasaki M, Yamaura C, Ohara-Nemoto Y, Tajika S, Kodama Y, Ohya T, et al. Streptococcus anginosus infection in oral cancer and its infection route. *Oral Dis* (2005) 11(3):151–6.
131. Mager DL, Haffajee AD, Devlin PM, Norris CM, Posner MR, Goodson JM. The salivary microbiota as a diagnostic indicator of oral cancer: a descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects. *J Trans Med* (2005) 3:27. doi: 10.1186/1479-5876-3-27
132. Lee J, Roberts JS, Atanasova KR, Chowdhury N, Han K, Yilmaz Ö. Human Primary Epithelial Cells Acquire an Epithelial-Mesenchymal-Transition Phenotype during Long-Term Infection by the Oral Opportunistic Pathogen, Porphyromonas gingivalis. *Front Cell Infect Microbiol* (2017) 7:493. doi: 10.3389/fcimb.2017.00493
133. Pushalkar S, Ji X, Li Y, Estilo C, Yegnanarayana R, Singh B, et al. Comparison of oral microbiota in tumor and non-tumor tissues of patients with oral squamous cell carcinoma. *BMC Microbiol* (2012) 12(1):1–15.
134. Wang H, Funchain P, Bebek G, Altemus J, Zhang H, Niazi F, et al. Microbiomic differences in tumor and paired-normal tissue in head and neck squamous cell carcinomas. *Genome Med* (2017) 9(1):1–10.
135. Zhang Z, Yang J, Feng Q, Chen B, Li M, Liang C, et al. Compositional and functional analysis of the microbiome in tissue and saliva of oral squamous cell carcinoma. *Front Microbiol* (2019) 10:1439.
136. Zhao H, Chu M, Huang Z, Yang X, Ran S, Hu B, et al. Variations in oral microbiota associated with oral cancer. *Sci Rep* (2017) 7:11773. doi: 10.1038/s41598-017-11779-9
137. Hooper SJ, Crean SJ, Lewis MA, Spratt DA, Wade WG, Wilson MJ. Viable bacteria present within oral squamous cell carcinoma tissue. *J Clin Microbiol* (2006) 44(5):1719–25.
138. Katz J, Onate MD, Pauley KM, Bhattacharyya I, Cha S. Presence of Porphyromonas gingivalis in gingival squamous cell carcinoma. *Int J Oral Sci* (2011) 3(4):209–15.
139. Sakamoto H, Naito H, Ohta Y, Tanakna R, Maeda N, Sasaki J, et al. Isolation of bacteria from cervical lymph nodes in patients with oral cancer. *Arch Oral Biol* (1999) 44(10):789–93.

140. Nagy K, Sonkodi I, Szöke I, Nagy E, Newman H. The microflora associated with human oral carcinomas. *Oral Oncol* (1998) 34(4):304–8.
141. Amer A, Galvin S, Healy CM, Moran GP. The microbiome of potentially malignant oral leukoplakia exhibits enrichment for Fusobacterium, Leptotrichia, Campylobacter, and Rothia species. *Front Microbiol* (2017) 8:2391.
142. Galvão-Moreira LV, da Cruz MCFN. Oral microbiome, periodontitis and risk of head and neck cancer. *Oral Oncol* (2016) 53:17–9. doi: 10.1016/j.oraloncology.2015.11.013
143. Karmakar S, Kar A, Thakur S, Rao VUS. Periodontitis and oral Cancer-A striking link. *Oral Oncol* (2020) 106:104630. doi: 10.1016/j.oraloncology.2020.104630
144. Hajishengallis G. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. *Trends Immunol* (2014) 35:3–11. doi: 10.1016/j.it.2013.09.001
145. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol* (2015) 15:30–44. doi: 10.1038/nri3785
146. Fitzpatrick SG, Katz J. The association between periodontal disease and cancer: a review of the literature. *J Dent* (2010) 38:83–95. doi: 10.1016/j.jdent.2009.10.007
147. Javed F, Warnakulasuriya S. Is there a relationship between periodontal disease and oral cancer? A systematic review of currently available evidence. *Crit Rev Oncol Hematol* (2015) 97:197–205. doi: 10.1016/j.critrevonc.2015.08.018
148. Li Y, Tan X, Zhao X, Xu Z, Dai W, Duan W, et al. Composition and function of oral microbiota between gingival squamous cell carcinoma and periodontitis. *Oral Oncol* (2020) 107:104710. doi: 10.1016/j.oraloncology.2020.104710
149. Su S-C, Chang L-C, Huang H-D, Peng C-Y, Chuang C-Y, Chen Y-T, et al. Oral microbial dysbiosis and its performance in predicting oral cancer. *Carcinogenesis* (2020) 1–9. doi: 10.1093/carcin/bgaa062
150. Kakabadze MZ, Paresishvili T, Karalashvili L, Chakhunashvili D, Kakabadze Z. Oral microbiota and oral cancer: Review. *Oncol Rev* (2020) 14:476. doi: 10.4081/oncol.2020.476
151. Perera M, Al-Hebshi NN, Perera I, Ipe D, Ulett GC, Speicher DJ, et al. Inflammatory Bacteriome and Oral Squamous Cell Carcinoma. *J Dent Res* (2018) 97:725–32. doi: 10.1177/0022034518767118
152. Al-Hebshi NN, Nasher AT, Idris AM, Chen T. Robust species taxonomy assignment algorithm for 16S rRNA NGS reads: application to oral carcinoma samples. *J Oral Microbiol* (2015) 7:28934. doi: 10.3402/jom.v7.28934
153. Stashenko P, Yost S, Choi Y, Danciu T, Chen T, Yoganathan S, et al. The Oral Mouse Microbiome Promotes Tumorigenesis in Oral Squamous Cell Carcinoma. *mSystems* (2019) 4:e00323–19. doi: 10.1128/mSystems.00323-19
154. Geng F, Liu J, Guo Y, Li C, Wang H, Wang H, et al. Persistent Exposure to Porphyromonas gingivalis Promotes Proliferative and Invasion Capabilities, and Tumorigenic Properties of Human Immortalized Oral Epithelial Cells. *Front Cell Infect Microbiol* (2017) 7:57. doi: 10.3389/fcimb.2017.00057
155. Ha NH, Woo BH, Kim DJ, Ha ES, Choi JI, Kim SJ, et al. Prolonged and repetitive exposure to Porphyromonas gingivalis increases aggressiveness of oral cancer cells by promoting acquisition of cancer stem cell properties. *Tumour Biol* (2015) 36:9947–60. doi: 10.1007/s13277-015-3764-9
156. Ha NH, Park DG, Woo BH, Kim DJ, Choi JI, Park BS, et al. Porphyromonas gingivalis increases the invasiveness of oral cancer cells by upregulating IL-8 and MMPs. *Cytokine* (2016) 86:64–72. doi: 10.1016/j.cyto.2016.07.013
157. Wen L, Mu W, Lu H, Wang X, Fang J, Jia Y, et al. Porphyromonas gingivalis Promotes Oral Squamous Cell Carcinoma Progression in an Immune Microenvironment. *J Dental Res* (2020) 99(6):666–675. doi: 10.1177/0022034520909312
158. Sztukowska MN, Ojo A, Ahmed S, Carenbauer AL, Wang Q, Shumway B, et al. Porphyromonas gingivalis initiates a mesenchymal-like transition through ZEB1 in gingival epithelial cells. *Cell Microbiol* (2015) 18(6):844–58. doi: 10.1111/cmi.12554
159. Inaba H, Sugita H, Kuboniwa M, Iwai S, Hamada M, Noda T, et al. Porphyromonas gingivalis promotes invasion of oral squamous cell carcinoma through induction of proMMP9 and its activation. *Cell Microbiol* (2014) 16:131–45. doi: 10.1111/cmi.12211
160. Binder Gallimidi A, Fischman S, Revach B, Bulvik R, Maliutina A, Rubinstein AM, et al. Periodontal pathogens Porphyromonas gingivalis and Fusobacterium nucleatum promote tumor progression in an oral-specific chemical carcinogenesis model. *Oncotarget* (2015) 6:22613–23. doi: 10.18632/oncotarget.4209
161. Lafuente Ibáñez de Mendoza I, Maritxalar Mendia X, García de la Fuente AM, Quindós Andrés G, Aguirre Urizar JM. Role of Porphyromonas gingivalis in oral squamous cell carcinoma development: A systematic review. *J Periodont Res* (2020) 55:13–22. doi: 10.1111/jre.12691
162. Yamamura K, Baba Y, Nakagawa S, Mima K, Miyake K, Nakamura K, et al. Human Microbiome Fusobacterium Nucleatum in Esophageal Cancer Tissue Is Associated with Prognosis. *Clin Cancer Res* (2016) 22:5574–81. doi: 10.1158/1078-0432.CCR-16-1786
163. Abdulkareem AA, Shelton RM, Landini G, Cooper PR, Milward MR. Potential role of periodontal pathogens in compromising epithelial barrier function by inducing epithelial-mesenchymal transition. *J Periodont Res* (2018) 53:565–74. doi: 10.1111/jre.12546
164. Zhang S, Li C, Liu J, Geng F, Shi X, Li Q, et al. Fusobacterium nucleatum promotes epithelial-mesenchymal transiton through regulation of the lncRNA MIR4435-2HG/miR-296-5p/Akt2/SNAI1 signaling pathway. *FEBS J* (2020) 287(18):4032–47. doi: 10.1111/febs.15233
165. Booijink CCGM, Boekhorst J, Zoetendal EG, Smidt H, Kleerebezem M, de Vos WM. Metatranscriptome Analysis of the Human Fecal Microbiota Reveals Subject-Specific Expression Profiles, with Genes Encoding Proteins Involved in Carbohydrate Metabolism Being Dominantly Expressed. *Appl Environ Microbiol* (2010) 76:5533–40. doi: 10.1128/AEM.00502-10
166. Duran-Pinedo AE, Chen T, Teles R, Starr JR, Wang X, Krishnan K, et al. Community-wide transcriptome of the oral microbiome in subjects with and without periodontitis. *ISME J* (2014) 8:1659–72. doi: 10.1038/ismej.2014.23
167. Jorth P, Turner KH, Gumus P, Nizam N, Buduneli N, Whiteley M. Metatranscriptomics of the Human Oral Microbiome during Health and Disease. *mBio* (2014) 5:e01012–14. doi: 10.1128/mBio.01012-14
168. Yost S, Duran-Pinedo AE, Teles R, Krishnan K, Frias-Lopez J. Functional signatures of oral dysbiosis during periodontitis progression revealed by microbial metatranscriptome analysis. *Genome Med* (2015) 7:27. doi: 10.1186/s13073-015-0153-3
169. Yost S, Stashenko P, Choi Y, Kukuruzinska M, Genco CA, Salama A, et al. Increased virulence of the oral microbiome in oral squamous cell carcinoma revealed by metatranscriptome analyses. *Int J Oral Sci* (2018) 10:32. doi: 10.1038/s41368-018-0037-7
170. Ma W, Mao Q, Xia W, Dong G, Yu C, Jiang F. Gut Microbiota Shapes the Efficiency of Cancer Therapy. *Front Microbiol* (2019) 10:1050. doi: 10.3389/fmicb.2019.01050
171. Panebianco C, Andriulli A, Pazienza V. Pharmacomicrobiomics: exploiting the drug-microbiota interactions in anticancer therapies. *Microbiome* (2018) 6:92. doi: 10.1186/s40168-018-0483-7
172. Fessler J, Matson V, Gajewski TF. Exploring the emerging role of the microbiome in cancer immunotherapy. *J Immunother Cancer* (2019) 7:108. doi: 10.1186/s40425-019-0574-4
173. Cremonesi E, Governa V, Garzon JFG, Mele V, Amicarella F, Muraro MG, et al. Gut microbiota modulate T cell trafficking into human colorectal cancer. *Gut* (2018) 67:1984–94. doi: 10.1136/gutjnl-2016-313498
174. Iida N, Dzutsev A, Stewart CA, Smith L, Bouladoux N, Weingarten RA, et al. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. *Science* (2013) 342:967–70. doi: 10.1126/science.1240527
175. Heshiki Y, Vazquez-Uribe R, Li J, Ni Y, Quainoo S, Imamovic L, et al. Predictable modulation of cancer treatment outcomes by the gut microbiota. *Microbiome* (2020) 8:28. doi: 10.1186/s40168-020-00811-2
176. Viaud S, Saccheri F, Mignot G, Yamazaki T, Daillère R, Hannani D, et al. The Intestinal Microbiota Modulates the Anticancer Immune Effects of Cyclophosphamide. *Science* (2013) 342:971–6. doi: 10.1126/science.1240537
177. Jenq RR, Taur Y, Devlin SM, Ponce DM, Goldberg JD, Ahr KF, et al. Intestinal Blautia Is Associated with Reduced Death from Graft-versus-Host Disease. *Biol Blood Marrow Transplant* (2015) 21:1373–83. doi: 10.1016/j.bbmt.2015.04.016

178. Peled JU, Devlin SM, Staffas A, Lumish M, Khanin R, Littmann ER, et al. Intestinal Microbiota and Relapse After Hematopoietic-Cell Transplantation. *J Clin Oncol* (2017) 35:1650–9. doi: 10.1200/JCO.2016.70.3348
179. Roy S, Trinchieri G. Microbiota: a key orchestrator of cancer therapy. *Nat Rev Cancer* (2017) 17:271–85. doi: 10.1038/nrc.2017.13
180. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. *Annu Rev Immunol* (2013) 31:51–72. doi: 10.1146/annurev-immunol-032712-100008
181. Touchefeu Y, Montassier E, Nieman K, Gastinne T, Potel G, Bruley des Varannes S, et al. Systematic review: the role of the gut microbiota in chemotherapy- or radiation-induced gastrointestinal mucositis - current evidence and potential clinical applications. *Aliment Pharmacol Ther* (2014) 40:409–21. doi: 10.1111/apt.12878
182. Barker HE, Paget JTE, Khan AA, Harrington KJ. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. *Nat Rev Cancer* (2015) 15:409–25. doi: 10.1038/nrc3958
183. Crawford PA, Gordon JI. Microbial regulation of intestinal radiosensitivity. *Proc Natl Acad Sci U S A* (2005) 102:13254–9. doi: 10.1073/pnas.0504830102
184. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer* (2013) 13:714–26. doi: 10.1038/nrc3599
185. Burt RK, Balabanov R, Burman J, Sharrack B, Snowden JA, Oliveira MC, et al. Effect of Nonmyeloablative Hematopoietic Stem Cell Transplantation vs Continued Disease-Modifying Therapy on Disease Progression in Patients With Relapsing-Remitting Multiple Sclerosis: A Randomized Clinical Trial. *JAMA* (2019) 321:165–74. doi: 10.1001/jama.2018.18743
186. Nilsson MS, Hallner A, Brune M, Nilsson S, Thorén FB, Martner A, et al. Complete remission after the first cycle of induction chemotherapy determines the clinical efficacy of relapse-preventive immunotherapy in acute myeloid leukaemia. *Br J Haematol* (2020) 188:e49–53. doi: 10.1111/bjh.16320
187. Bruna R, Benedetti F, Boccomini C, Patti C, Barbui AM, Pulsoni A, et al. Prolonged survival in the absence of disease-recurrence in advanced-stage follicular lymphoma following chemo-immunotherapy: 13-year update of the prospective, multicenter randomized GITMO-IIL trial. *Haematologica* (2019) 104:2241–8. doi: 10.3324/haematol.2018.209932
188. Haddad R, Concha-Benavente F, Blumenschein G, Fayette J, Guigay J, Colevas AD, et al. Nivolumab treatment beyond RECIST-defined progression in recurrent or metastatic squamous cell carcinoma of the head and neck in CheckMate 141: A subgroup analysis of a randomized phase 3 clinical trial. *Cancer* (2019) 125:3208–18. doi: 10.1002/cncr.32190
189. Paz-Ares L, Dvorkin M, Chen Y, Reinmuth N, Hotta K, Trukhin D, et al. Durvalumab plus platinum-etoposide versus platinum-etoposide in first-line treatment of extensive-stage small-cell lung cancer (CASPIAN): a randomised, controlled, open-label, phase 3 trial. *Lancet* (2019) 394:1929–39. doi: 10.1016/S0140-6736(19)32222-6
190. Nixon AB, Schalper KA, Jacobs I, Potluri S, Wang I-M, Fleener C. Peripheral immune-based biomarkers in cancer immunotherapy: can we realize their predictive potential? *J Immunother Cancer* (2019) 7:325. doi: 10.1186/s40425-019-0799-2
191. Wilky BA. Immune checkpoint inhibitors: The linchpins of modern immunotherapy. *Immunol Rev* (2019) 290:6–23. doi: 10.1111/imr.12766
192. Marcus L, Lemery SJ, Keegan P, Pazdur R. FDA Approval Summary: Pembrolizumab for the Treatment of Microsatellite Instability-High Solid Tumors. *Clin Cancer Res* (2019) 25:3753–8. doi: 10.1158/1078-0432.CCR-18-4070
193. Vétizou M, Pitt JM, Daillère R, Lepage P, Waldschmitt N, Flament C, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* (2015) 350(6264):1079–84.
194. Chaput N, Lepage P, Coutzac C, Soularue E, Le Roux K, Monot C, et al. Baseline gut microbiota predicts clinical response and colitis in metastatic melanoma patients treated with ipilimumab. *Ann Oncol* (2017) 28:1368–79. doi: 10.1093/annonc/mdx108
197. Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, et al. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science* (2015) 350:1084–9. doi: 10.1126/science.aac4255
195. Matson V, Fessler J, Bao R, Chongsuwat T, Zha Y, Alegre M-L, et al. The commensal microbiome is associated with anti–PD-1 efficacy in metastatic melanoma patients. *Science* (2018) 359(6371):104–8.
196. Routy B, Le Chatelier E, Derosa L, Duong CPM, Alou MT, Daillère R, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* (2018) 359:91–7. doi: 10.1126/science.aan3706
198. Naidoo J, Page DB, Li BT, Connell LC, Schindler K, Lacouture ME, et al. Toxicities of the anti-PD-1 and anti-PD-L1 immune checkpoint antibodies. *Ann Oncol* (2015) 26:2375–91. doi: 10.1093/annonc/mdv383
199. Tanoue T, Morita S, Plichta DR, Skelly AN, Suda W, Sugiura Y, et al. A defined commensal consortium elicits CD8 T cells and anti-cancer immunity. *Nature* (2019) 565:600–5. doi: 10.1038/s41586-019-0878-z
200. Gopalakrishnan V, Helmink BA, Spencer CN, Reuben A, Wargo JA. The influence of the gut microbiome on cancer, immunity, and cancer immunotherapy. *Cancer Cell* (2018) 33:570–80. doi: 10.1016/j.ccell.2018.03.015
201. Fong W, Li Q, Yu J. Gut microbiota modulation: a novel strategy for prevention and treatment of colorectal cancer. *Oncogene* (2020) 39:4925–43. doi: 10.1038/s41388-020-1341-1
202. Dubin K, Callahan MK, Ren B, Khanin R, Viale A, Ling L, et al. Intestinal microbiome analyses identify melanoma patients at risk for checkpoint-blockade-induced colitis. *Nat Commun* (2016) 7:10391. doi: 10.1038/ncomms10391
203. Yu T, Guo F, Yu Y, Sun T, Ma D, Han J, et al. Fusobacterium nucleatum Promotes Chemoresistance to Colorectal Cancer by Modulating Autophagy. *Cell* (2017) 170:548–563.e16. doi: 10.1016/j.cell.2017.07.008
204. Pawelek JM, Low KB, Bermudes D. Bacteria as tumour-targeting vectors. *Lancet Oncol* (2003) 4:548–56. doi: 10.1016/s1470-2045(03)01194-x
205. Fehleisen F. Die Aetiologie des Erysipels. *Theodor Fischer* (1883).
206. Coley WBII. Contribution to the Knowledge of Sarcoma. *Ann Surg* (1891) 14:199–220. doi: 10.1097/00000658-189112000-00015
207. McCarthy EF. The Toxins of William B. Coley and the Treatment of Bone and Soft-Tissue Sarcomas. *Iowa Orthop J* (2006) 26:154–8.
208. Mengesha A, Dubois L, Chiu RK, Paesmans K, Wouters BG, Lambin P, et al. Potential and limitations of bacterial-mediated cancer therapy. *Front Biosci* (2007) 12:3880–91. doi: 10.2741/2357
209. Nallar SC, Xu D-Q, Kalvakolanu DV. Bacteria and genetically modified bacteria as cancer therapeutics: Current advances and challenges. *Cytokine* (2017) 89:160–72. doi: 10.1016/j.cyto.2016.01.002
210. Yazawa K, Fujimori M, Amano J, Kano Y, Taniguchi S. Bifidobacterium longum as a delivery system for cancer gene therapy: selective localization and growth in hypoxic tumors. *Cancer Gene Ther* (2000) 7:269–74. doi: 10.1038/sj.cgt.7700122
211. Parker RC, Plummer HC. Effect of histolyticus infection and toxin on transplantable mouse tumors. *Proc Soc Exp Biol Med* (1947) 66:461–7. doi: 10.3181/00379727-66-16124
212. Moese JR, Moese G. Oncolysis by clostridia. I. Activity of clostridium butyricum (m-55) and other nonpathogenic clostridia against the ehrlich carcinoma. *Cancer Res* (1964) 24:212–6.
213. Theys J, Landuyt W, Nuyts S, Van Mellaert L, van Oosterom A, Lambin P, et al. Specific targeting of cytosine deaminase to solid tumors by engineered Clostridium acetobutylicum. *Cancer Gene Ther* (2001) 8:294–7. doi: 10.1038/sj.cgt.7700303
214. Guo Y, Chen Y, Liu X, Min J-J, Tan W, Zheng JH. Targeted cancer immunotherapy with genetically engineered oncolytic Salmonella typhimurium. *Cancer Lett* (2020) 469:102–10. doi: 10.1016/j.canlet.2019.10.033
215. Liang K, Liu Q, Li P, Luo H, Wang H, Kong Q. Genetically engineered Salmonella Typhimurium: Recent advances in cancer therapy. *Cancer Lett* (2019) 448:168–81. doi: 10.1016/j.canlet.2019.01.037
216. Cunningham C, Nemunaitis J. A phase I trial of genetically modified Salmonella typhimurium expressing cytosine deaminase (TAPET-CD, VNP20029) administered by intratumoral injection in combination with 5-fluorocytosine for patients with advanced or metastatic cancer. Protocol no: CL-017. Version: April 9, 2001. *Human Gene Therap* (2001) 12(2):1594–6. doi: 10.1371/journal.pone.0209153
217. Chen W, Wang Y, Qin M, Zhang X, Zhang Z, Sun X, et al. Bacteria-Driven Hypoxia Targeting for Combined Biotherapy and Photothermal

Therapy. *ACS Nano* (2018) 12:5995–6005. doi: 10.1021/acsnano.8b02235
218. Dai Z, Zhang J, Wu Q, Fang H, Shi C, Li Z, et al. Intestinal microbiota: a new force in cancer immunotherapy. *Cell Commun Signal* (2020) 18:90. doi: 10.1186/s12964-020-00599-6
219. Zebertavage L, Bambina S, Shugart J, Alice A, Zens KD, Lauer P, et al. A microbial-based cancer vaccine for induction of EGFRvIII-specific CD8+ T cells and anti-tumor immunity. *PLoS One* (2019) 14:e0209153. doi: 10.1371/journal.pone.0209153
220. D'Haens GR, Jobin C. Fecal Microbial Transplantation for Diseases Beyond Recurrent Clostridium Difficile Infection. *Gastroenterology* (2019) 157:624–36. doi: 10.1053/j.gastro.2019.04.053
221. Kitamoto S, Nagao-Kitamoto H, Hein R, Schmidt TM, Kamada N. The Bacterial Connection between the Oral Cavity and the Gut Diseases. *J Dent Res* (2020) 99:1021–9. doi: 10.1177/0022034520924633
222. Kitamoto S, Nagao-Kitamoto H, Jiao Y, Gillilland MG, Hayashi A, Imai J, et al. The Intermucosal Connection between the Mouth and Gut in Commensal Pathobiont-Driven Colitis. *Cell* (2020) 182:447–462.e14. doi: 10.1016/j.cell.2020.05.048

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O-Polysaccharide Plays a Major Role on the Virulence and Immunostimulatory Potential of *Aggregatibacter actinomycetemcomitans* During Periodontal Infection

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Aggregatibacter actinomycetemcomitans is a Gram-negative oral bacterium with high immunostimulatory and pathogenic potential involved in the onset and progression of periodontitis, a chronic disease characterized by aberrant immune responses followed by tooth-supporting bone resorption, which eventually leads to tooth loss. While several studies have provided evidence related to the virulence factors of *A. actinomycetemcomitans* involved in the host cell death and immune evasion, such as its most studied primate-specific virulence factor, leukotoxin, the role of specific lipopolysaccharide (LPS) domains remain poorly understood. Here, we analyzed the role of the immunodominant domain of the LPS of *A. actinomycetemcomitans* termed O-polysaccharide (O-PS), which differentiates the distinct bacterial serotypes based on its antigenicity. To determine the role of the O-PS in the immunogenicity and virulence of *A. actinomycetemcomitans* during periodontitis, we analyzed the *in vivo* and *in vitro* effect of an O-PS-defective transposon mutant serotype *b* strain, characterized by the deletion of the *rmlC* gene encoding the α-L-rhamnose sugar biosynthetic enzyme. Induction of experimental periodontitis using the O-PS-defective *rmlC* mutant strain resulted in lower tooth-supporting bone resorption, infiltration of Th1, Th17, and Th22 lymphocytes, and expression of *Ahr*, *Il1b*, *Il17*, *Il23*, *Tlr4*, and RANKL (*Tnfsf11*) in the periodontal lesions as compared with the wild-type *A. actinomycetemcomitans* strain. In addition, the O-PS-defective *rmlC* mutant strain led to impaired activation of antigen-presenting cells, with less expression of the co-stimulatory molecules CD40 and CD80 in B lymphocytes and dendritic cells, and downregulated expression of *Tnfa* and *Il1b* in splenocytes. In

conclusion, these data demonstrate that the O-PS from the serotype *b* of *A. actinomycetemcomitans* plays a key role in the capacity of the bacterium to prime oral innate and adaptive immune responses, by triggering the Th1 and Th17-driven tooth-supporting bone resorption during periodontitis.

Keywords: periodontitis, *Aggregatibacter actinomycetemcomitans*, lipopolysaccharide, LPS, O-polysaccharide, O-PS, T-lymphocytes, bone resorption

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a small, non-motile, Gram-negative coccobacillus, resident in the oral cavity of humans and non-human primates, that preferentially colonizes the tissues that surround teeth (1). *A. actinomycetemcomitans* is strongly implicated in the pathogenesis of periodontitis, an inflammatory disease characterized by the disruption of the equilibrium among the periodontal microbiota and the host's immune response, that subsequently leads to an increased local Th1 and Th17-type of response. Indeed, *A. actinomycetemcomitans* is considered a key player of the pathogenic consortium related to severe periodontitis, which causes accelerated periodontal tissue breakdown, in particular, the Th17-driven tooth-supporting bone resorption, the major pathological sign related with tooth loss (2, 3).

Alveolar bone resorption is the hallmark of periodontitis. During homeostatic conditions, the bone remodeling process is continuously maintained by the coupled action of bone-resorptive osteoclasts and bone-forming osteoblasts in a fine-tuned equilibrium regulated by a triad of molecules: the receptor activator of nuclear-factor κB ligand (RANKL), also known as tumor necrosis factor ligand superfamily member 11 (*Tnfsf11*), its specific receptor (RANK), and its soluble decoy osteoprotegerin (OPG) (4, 5). Indeed, RANKL is a key molecule involved in osteoclastogenesis and osteoclast-mediated bone resorption by stimulating the osteoclast progenitor differentiation and mature osteoclast activity (5). Otherwise, the immune response triggered against the dysbiotic subgingival microbiota during periodontitis disturbs the osteoblast/osteoclast equilibrium by dramatically increasing the RANKL local production and cellular sources, leading to pathological alveolar bone resorption (6, 7). Initial subversion of the host's immunity by periodontal keystone pathogens and other oral bacteria promotes the accumulation of immunostimulatory pathobionts, such as *A. actinomycetemcomitans*, that finally contribute to immune-mediated alveolar bone loss (6–8).

Clinical evidence suggests a variable virulence potential among the distinct serotypes of *A. actinomycetemcomitans* (2, 9–11). Currently, seven *A. actinomycetemcomitans* serotypes are recognized based on the antigenicity of the O-polysaccharide (O-PS) immunodominant component of their lipopolysaccharide (LPS), being the serotypes *a*, *b*, and *c* the most frequently detected in humans (12–14). *A. actinomycetemcomitans* strains belonging to the serotype *b* are frequently isolated from subjects with severe periodontitis, while serotypes *a* and *c* are mostly isolated from milder periodontitis-affected patients and healthy individuals (2, 15–17). This variable pathological association has been related to the increased virulence of the serotype *b*, as we recently reported using an animal model of periodontitis, in which a higher lymphocyte Th1 and Th17-driven alveolar bone resorption was observed when the serotype *b* was used for periodontitis induction, as compared with the serotypes *a* or *c* of *A. actinomycetemcomitans* (18). Besides, *in vitro* studies have ratified the higher immunostimulatory potential of serotype *b* of *A. actinomycetemcomitans* on human antigen-presenting cells and T lymphocytes, as compared with the other serotypes (19–21), as well as its increased leukotoxin production (22). Indeed, most of the studies that analyze the higher virulence of serotype b of *A. actinomycetemcomitans* have focused on the production of leukotoxin, an exotoxin that selectively induces cell death in hematopoietic cells of human and non-human primate origin (23); however, the role of its O-PS has been scarcely studied.

The O-PS from *A. actinomycetemcomitans* serotype *b* is structurally distinct from the O-PS from the other serotypes. The O-PS from serotype *b* is composed of a disaccharide backbone of α-D-fucose (D-Fuc) and α-L-rhamnose (L-Rha), linked by a non-reducing β-D-N-acetyl-galactosamine (D-GalNAc) residue, and the O-PS from serotypes *a* and *c* consists of 6-deoxy- α-D-talose (α-D-Tal) and 6-deoxy-α-L-talose (α-L-Tal), respectively (24, 25). In the case of serotype *b*, the enzyme TDP-4-keto-6-deoxy-D-glucose 3,5-epimerase (RmlC) is required for the L-Rha synthesis and its assembling to the O-PS structure, and the genetic deletion of the *rmlC* gene encoding this enzyme completely abolish the production of the O-PS moiety, leading to a generation of an O-PS-defective *A. actinomycetemcomitans* strain (26). The *A. actinomycetemcomitans* strain lacking O-PS has shown altered bacterial adhesion and decreased leukotoxin secretion (26, 27); however, its pathogenic and immunostimulatory potential has not been analyzed yet.

In this study, we analyzed the role of the O-PS in the immunogenicity and virulence of *A. actinomycetemcomitans*. We took advantage of a murine model of periodontitis infected with the *rmlC* mutant strain, belonging to the serotype *b*. The deletion of the *rmlC* gene generated an O-PS-defective strain with reduced virulence, as demonstrated by the decreased tooth-supporting bone resorption in infected mice. This decreased alveolar bone loss was associated with downregulated levels of Th1 and Th17-related cytokines and less infiltration of Th1 and Th17 lymphocytes within the periodontal lesions. Besides, this O-PS-defective *A. actinomycetemcomitans* strain triggered a downregulated expression of *Tnfa* and *Il1b* in splenocytes and co-stimulatory molecules CD40 and CD80 in B lymphocytes and dendritic cells, in comparison to the wild-type and complemented strains. Collectively, these results demonstrate that the O-PS moiety from the serotype *b* of *A. actinomycetemcomitans* plays a key role in its

immunostimulatory and virulent potential, affecting the maturation of antigen-presenting cells and the Th1 and Th17-driven alveolar bone resorption during periodontitis.

MATERIALS AND METHODS

A. actinomycetemcomitans Strains

The *A. actinomycetemcomitans* mutant strains used in the current study are based on the nonfimbriated VT1169 strain belonging to the serotype *b* (all of them kindly provided by Dr. Keith Mintz, Department of Microbiology and Molecular Genetics, University of Vermont). In order to analyze the role of O-PS of *A. actinomycetemcomitans*, two mutant strains were used: the *rmlC* mutant strain, characterized by the absence of detectable O-PS, and the *waaL* mutant strain, characterized by impaired production of O-PS compared with the VT1169 wild-type strain, which was used for comparison (26). The transposon-generated *rmlC* mutant strain and the *waaL* mutant strain, generated by site-directed insertional mutagenesis, have been previously characterized (26). The *rmlC/rmlC*$^+$ and *waaL/waaL*$^+$ complemented strains, characterized by the restoration of the production of O-PS in a similar profile to the VT1169 wild-type strain, were used as controls (26). The *A. actinomycetemcomitans* strains were grown in 3% trypticase soy broth 0.6% yeast extract (TBSYE; Oxoid Ltd., England), supplemented with or without specific antibiotics, at 37°C and under capnophilic conditions. The *rmlC* and *waaL* mutant strains were grown in the presence of 50 μg/ml spectinomycin, and the *rmlC/rmlC*$^+$ and *waaL/waaL*$^+$ complemented strains were grown in the presence of 1 μg/ml chloramphenicol. To obtain a reliable number of live bacteria with their whole antigenic potentiality for the periodontitis induction and cell stimulation, growth curves were made and bacteria were obtained at the exponential growth phase as previously described (28, 29). All the mutant and complemented *A. actinomycetemcomitans* strains used in the present study kept similar growth characteristics and colony morphology to the wild-type VT1169 strain.

Lipopolysaccharide (LPS) Purification and Analysis

The LPS of the studied *A. actinomycetemcomitans* strains was purified using a modified hot phenol extraction protocol, based on a previously described protocol (30). LPS was visualized by Tricine-SDS-PAGE gel with 14% acrylamide/bis-acrylamide 46.5:3 solution, stained with a silver solution, as previously described (31). The gel was revealed in a solution containing 12.5 mg citric acid and 125 μL formaldehyde 37% in 250 mL of deionized water, photo-documented on a transilluminator, and analyzed using the Molecular Imaging Software "MI" 7.2 win (Bruker, Belgium).

Animals

For periodontitis induction, eight-week-old wild-type BALB/c female mice were obtained at the estrus stage of the estrous cycle, to avoid the potential influence of sex hormones on bone metabolism. For *in vitro* assays, spleen cells were obtained from eight to sixteen-weeks-old C57BL/6 background mice. Animals were housed in separate cages and maintained under standard conditions: a 12:12 h light/dark cycle, lights on at 07:00 am, at 24°C ± 0.5°C, and 40% to 70% of relative humidity, with an air exchange rate of 15-room volumes/hour. Throughout the period of the study, animals had free access to sterile standard solid mice chow and water. Animals were handled according to the protocol approved by the Institutional Committee for Animal Care and Use from Universidad de Chile (Ethical permit #061601) and the Stockholm Regional Ethics Committee.

Periodontal Infection

Periodontal infections were induced following a previously described protocol (18). Under ketamine/xylazine anesthesia, 2 μl of phosphate-buffered saline (PBS) cell suspension containing 1x10^9 CFU/ml of each *A. actinomycetemcomitans* strain were microinjected using a 26s-gauge syringe (Hamilton, USA). A total of three microinjections were performed every 48 h into the palatal interproximal gingiva between the first and second molar of the right and left side of the maxilla (**Figure 1A**). Animals were randomly allocated in seven groups with three to four mice in each group: (a) VT1169 strain-infected group, (b) *rmlC* mutant strain-infected group, (c) *rmlC/rmlC*$^+$ complemented strain-infected group, (d) *waaL* mutant strain-infected group, (e) *waaL/waaL*$^+$ complemented strain-infected group, (f) sham-infected mice, which received PBS without bacteria, and (g) untreated animals. The mice were euthanized after 30 days by a single overdose of ketamine/xylazine anesthesia, and samples of maxillae, palatal periodontal tissues, and cervical lymph nodes were collected for further analysis. No changes were detected in the body-weight of mice throughout the study (**Supplementary Figure S1**).

Quantification of Alveolar Bone Resorption

To quantify the extent of alveolar bone loss, the maxillae were scanned using a micro-computed tomography (μCT) equipment (SkyScan 1272; Bruker, Belgium), as previously described (18, 32). 3D-digitized images were generated using a modified cone-beam algorithm in a reconstruction software (Nrecon software, Bruker, Belgium). Alveolar bone loss was quantified using two methods: (a) by measuring the linear distance from the cement-enamel junction (CEJ) to the alveolar bone crest (ABC) and (b) by quantifying the percentage of remnant alveolar bone, by considering the average of untreated mice bone levels as 100%, as previously described (33, 34). In turn, the CEJ-ABC linear distance was quantified in (a) the interdental area comprised between the first and second molar and (b) the distal surface of the distal root of the first molar. All data were collected by a single observer (F.C.), who was masked to the conditions of the maxillae specimens.

qRT-PCR From Palatal Periodontal Tissues

From the palatal periodontal tissues, total cytoplasmic RNA was purified, as previously described (18). After its quantification in a

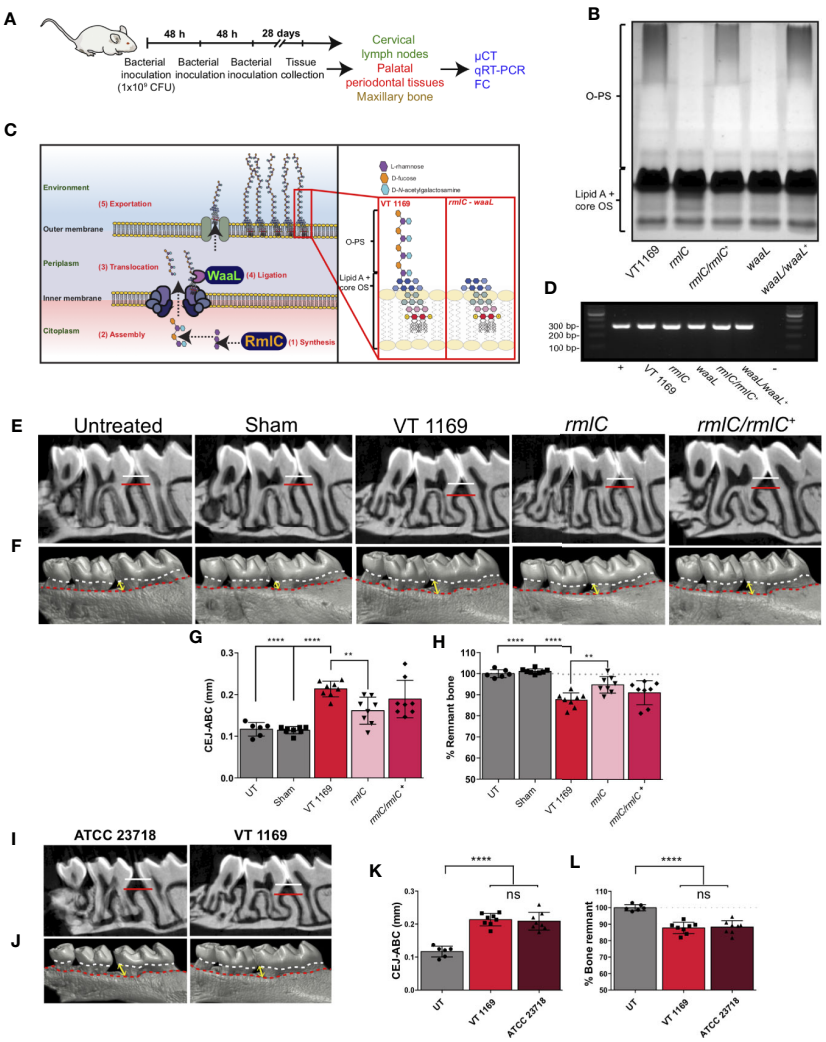

FIGURE 1 | Lipopolysaccharide (LPS) analysis and alveolar bone loss triggered by the different strains of *A. actinomycetemcomitans*. **(A)** Diagram illustrating the protocol for the bacteria-induced periodontitis model. **(B)** LPS profiles obtained by SDS-PAGE analysis of the VT1169 wild-type strain and the *rmlC* and *waaL* mutant strains, as well as the *rmlC/rmlC*$^+$ and *waaL/waaL*$^+$ complemented strains. **(C)** Schematic view of the *A. actinomycetemcomitans* serotype *b* O-polysaccharide (O-PS) biosynthetic pathway, highlighting the modified enzymes and the O-PS profile obtained by Tang et al. (26) and used in the present study. **(D)** The 2% agarose gel of the amplification products obtained with specific primers for the strain VT1169, from PCR analysis of the V3-V6 variable region of the 16S rDNA. **(E)** Sagittal slices of the 2D-reconstruction by µCT showing the alveolar bone loss determined as the CEJ-ABC linear distance in the interdental area between the first and second molar (white-red lines) in mice infected with the *A. actinomycetemcomitans* VT1169, *rmlC*, or *rmlC/rmlC*$^+$ strains and the sham-infected and untreated controls. **(F)** Vestibular view of the 3D-reconstruction by µCT showing the alveolar bone loss, determined as the CEJ-ABC linear distance in the distal surface of the distal root of the first molar in the same experimental conditions described in **(E)**. Yellow double-arrow lines represent the CEJ-ABC linear distance (CEJ marked as a dashed white line and ABC marked as a dashed red line). **(G)** Quantification of the alveolar bone loss determined as the CEJ-ABC linear distance in the distal surface of the distal root of the first molar in mice infected with the *A. actinomycetemcomitans* VT1169, *rmlC*, or *rmlC/rmlC*$^+$ strains and the sham-infected and untreated controls (UT). **(H)** Quantification of the alveolar bone loss determined as the percentage of remnant alveolar bone in mice infected with the *A. actinomycetemcomitans* VT1169, *rmlC*, or *rmlC/rmlC*$^+$ strains and the sham-infected and UT controls, considering the average of remnant alveolar bone in UT mice as 100%. **(I)** Sagittal slices of the 2D-reconstruction by µCT showing the alveolar bone loss determined as the CEJ-ABC linear distance in the interdental area between the first and second molar (white-red lines) in mice infected with the *A. actinomycetemcomitans* VT1169 and ATCC 23718 strains. **(J)** Vestibular view of the 3D-reconstruction by µCT showing the alveolar bone loss, determined as the CEJ-ABC linear distance in the distal surface of the distal root of the first molar in the same experimental conditions described in **(I)**. Yellow double-arrow lines represent the CEJ-ABC linear distance (CEJ marked as a dashed white line and ABC marked as a dashed red line). **(K)** Quantification of the alveolar bone loss determined as the CEJ-ABC linear distance in the distal surface of the distal root of the first molar in mice infected with the *A. actinomycetemcomitans* VT1169 and ATCC 23718 strains and UT controls. **(L)** Quantification of the alveolar bone loss determined as the percentage of remnant alveolar bone in mice infected with the *A. actinomycetemcomitans* VT1169 and ATCC 23718 strains and UT controls, considering the average of remnant alveolar bone in UT mice as 100%. Mean ± SD, one-way ANOVA and Tukey post-hoc test, $^{**}p < 0.01$, $^{****}p < 0.0001$. Error bars represent SEM in all panels. ABC, alveolar bone crest; CEJ, cement-enamel junction; CFU, colony-forming units; cLNs, cervical lymph nodes; FC, flow cytometry; ns, no significant; LPS, lipopolysaccharide; O-PS, O-polysaccharide; OS, oligosaccharide; PPTs, palatal periodontal tissues.

spectrophotometer (Synergy HT; Bio-Tek Instrument Inc., USA), the first-strand of cDNA was synthesized from 1 µg of total RNA using the SuperScript III kit (Invitrogen, USA), according to the manufacturer's instructions. In order to quantify the mRNA expression levels for *Ahr*, *Ifng*, *Il6*, *Il10*, *Il17*, *Il22*, *Il23*, *Il1b*, *Tlr2*, *Tlr4*, and RANKL (*Tnfsf11*), 10 ng of cDNA were amplified using the appropriate primers (**Supplementary Table S1**) and the KAPA SYBR Fast qPCR reagent (KAPA Biosystems, USA), in a qPCR apparatus (StepOnePlus; Applied Biosystems, Singapore). Amplification reactions were conducted as follows: a first denaturation step of 95°C for 3 min, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. For detection of non-specific product formation and false-positive amplification, a final melting curve of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s was performed. Fold change of mRNA expression was calculated relative to the undisturbed 18S rRNA expression levels, using the $2^{-\Delta\Delta Ct}$ method followed by a $\log_2$-transformation.

Single-Cell Suspension From Palatal Periodontal Tissues and Cervical Lymph Nodes

In order to obtain cells to perform the flow cytometric analysis, the palatal periodontal tissues and cervical lymph nodes that drain the periodontal tissues were processed, as previously described (32). For the recovery of palatal periodontal tissues, after accessing the oral cavity, whole maxillary blocks separated from the nasal cavity were dissected and immediately processed for enzymatic digestion. For this, specimens were immersed in 5 ml collagenase D/DNase (Sigma, USA) and incubated under constant agitation at 37°C for 1 hr. During the last 5 min of enzymatic digestion, specimens were incubated with 50 µl of 0.5 M EDTA (Sigma, USA) to stop the reaction. Then, in the presence of cold DNase media, the palatal periodontal tissues were removed from the maxillary bone, minced into 1 mm^3 pieces, and mechanically mashed using a 70-µm cell-strainer, to obtain a single-cell suspension. After washing twice with cold DNase media, live cells were counted using an automated cell counter (Luna II; Logos Biosystems, South Korea). For the draining cervical lymph nodes, the cell suspensions were obtained through mechanic disruption of the three bilateral cervical lymph nodes that drain the periodontal tissues (mandibular, accessory mandibular, and superficial parotid), using a 70-µm cell-strainer in the presence of cold PBS.

Flow Cytometry of Cells Obtained From Palatal Periodontal Tissues and Cervical Lymph Nodes

The presence of $Tbet^+$ (Th1), $ROR\gamma t^+$ (Th17), and AhR^+ (Th22) lymphocytes within the periodontal lesions and cervical lymph nodes were analyzed by identifying the expression of their specific transcription factors using flow cytometry, as previously described (18, 32). The identified transcription factors were T-bet for Th1 cells, RORγt for Th17 cells, and AhR for Th22 cells. For this, the obtained cells were incubated for 15 min at 4°C with a Fc-blocking (CD16/32) antibody (eBioscience, Thermo Fisher Scientific, USA) prior to staining with fluorochrome-conjugated antibodies. For extracellular staining, the following lineage cocktail was used: CD45 (30-f11), CD4 (GK1.5), and CD3 (17A2). For intracellular staining, cells were incubated with a Fixation and Permeabilization buffer (Fix/Perm kit, eBioscience, Thermo Fisher Scientific, USA) for 30 min at 4°C followed by staining overnight at 4°C with the following antibodies: T-bet (4B10), RORγt (4G419), or AhR (4MEJJ). In addition, to analyze the immune cell compartment, the following cocktail was used: CD45.2 (104), CD19 (6D5), MHC-II (M5/114.15.1), CD3 (145-2c11), CD90 (53-2.1), CD11c (N418), CD11b (M1/70), Ly6C (HK1.4), Ly6G (1A8), and CD64 (54-5/7.1) (eBioscience, Thermo Fisher Scientific, USA; Biolegend, USA; and Abcam, England). Live/Dead Fixable viability dyes (eBioscience, Thermo Fisher Scientific, USA) were used to exclude dead cells. All the experiments were acquired using a FACS LSR Fortessa (BD Biosciences, USA) and analyzed with the FlowJo software (BD Biosciences, USA).

Flow Cytometric Data Analysis by tSNE

T-distributed stochastic neighbor embedding (tSNE) analysis of 11-parameter flow cytometry data obtained from untreated mice was performed using the FlowJo software (BD Biosciences, USA). Samples were randomly downsampled to 10,000 events per sample, and analysis was run on equivalent numbers of events per sample. Following downsampling, 11-parameter samples were concatenated and visualized with tSNE (Barnes-Hut implementation) in FlowJo. The following parameters were tuned in preliminary experiments and then used as default values: iterations, 550; perplexity, 40; eta, 200; theta, 0.5. All parameters, except for lineage marker CD45 and DAPI live/dead marker, were included in the analysis.

Total Splenocytes *In Vitro* Assays

Splenocytes were harvested from C57BL/6 background mice and maintained in IMDM medium (Gibco), supplemented with heat-inactivated 10% fetal calf serum (Sigma, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2% beta-mercaptoethanol (Gibco, Thermo Fisher Scientific, USA). Splenocytes ($3x10^6$ cells/well) in 24-well plates were challenged with the *rmlC* or *waaL* mutant strains, the *rmlC*/*rmlC*$^+$ or *waaL*/*waaL*$^+$ complemented strains, or the VT1169 wild-type strain of *A. actinomycetemcomitans*, at a multiplicity of infection (MOI) 3 for 20 h for flow cytometric assays, or 8 h for qRT-PCR assays. Untreated cells or cells treated with 1 µg/ml of commercial *Escherichia coli*-derived LPS (Sigma, USA) were used as controls. The selected MOI was based on dose-response assays (**Supplementary Figure S2**).

qRT-PCR From Induced Splenocytes

After 8 h of cell stimulation, splenocytes were washed and preserved in RNAlater at -80°C. Then, after cells were homogenized using a syringe, the total cytoplasmic RNA was isolated using the RNAeasy Mini Kit (Qiagen, Germany), and the first-strand cDNA was synthesized using the iScript RT Supermix (BioRad, USA), according to the manufacturer's

instructions. In order to quantify the mRNA expression levels for *Il1b*, *Il17*, *Il23*, and *Tnfa*, for the qRT-PCR analysis, the appropriate primers (**Supplementary Table S1**) and the iTaq Universal SYBR Green Supermix reagent (BioRad, USA) were used. Amplification reactions were conducted as follows: a first denaturation step of 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 20 s. Fold change of mRNA expression was calculated relative to the *Hprt* expression levels, using the $2^{-\Delta\Delta Ct}$ method followed by a $\log_2$-transformation.

Flow Cytometry of Stimulated Splenocytes

In order to analyze the surface expression of co-stimulatory molecules CD40 and CD80 in B lymphocytes, dendritic cells, and macrophages obtained from total splenocytes, the following antibodies were used: CD19 (6D5), MHC-II (M5/114.15.1), CD11c (N418), CD11b (M1/70), Ly6C (HK1.4), CD80 (16-10A1), and CD40 (HM40-3) (eBioscience, Thermo Fisher Scientific, USA; Biolegend, USA; and Abcam, England). Live/Dead Fixable viability dyes (eBioscience, USA) were used to exclude dead cells. All the experiments were acquired using a FACS LSR Fortessa X20 flow cytometer (BD Biosciences, USA) and analyzed with FlowJo software (BD Biosciences, USA).

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism v.4.01 software (GraphPad Software, Inc., USA). Heatmaps from qRT-PCR fold-change data were done in Excel v.16.39 software (Microsoft, USA). The normality of data distribution was established using the Kolmogorov-Smirnov test. When a parametric analysis was carried out, the statistical differences were determined using the ANOVA and the Tukey or Holm-Sidak post-hoc tests. When a nonparametric analysis was carried out, the statistical differences were determined using the Kruskal-Wallis and Dunn tests. A difference was considered significant when $p < 0.05$.

RESULTS

Analysis of the LPS Purified From the Studied *A. actinomycetemcomitans* Mutant Strains

To confirm that the *A. actinomycetemcomitans* mutant strains used in this study were characterized by changes in the O-PS expression, we carried out the electrophoretic assays of their purified LPS. The silver-stained profile of the LPS obtained from both the *rmlC* and *waaL* mutant strains revealed the absence of the O-PS domain (**Figure 1B**), in accordance with the previously reported profiles (26) (**Figure 1C**). The *rmlC*/*rmlC*$^+$ and *waaL*/*waaL*$^+$ complemented strains displayed a restored LPS profile with the presence of a complete O-PS moiety (**Figure 1B**). All the bacterial strains used for periodontitis induction and cell challenge were positive for the *A. actinomycetemcomitans* serotype *b*, as demonstrated by the PCR analysis of the V3-V6 variable region of the 16S rDNA (**Figure 1D**).

The O-PS-Defective *A. actinomycetemcomitans* Serotype *b* Mutant Strain Induces Less Alveolar Bone Resorption Than the Wild-Type Strain

To elucidate the pathogenic role of the O-PS moiety from the *A. actinomycetemcomitans* serotype *b*, we used a previously described experimental animal model of periodontitis (18), and the extent of the alveolar bone resorption was determined using μCT (**Figures 1E, F**). Mice infected with the VT1169 wild-type strain of *A. actinomycetemcomitans* exhibited greater alveolar bone loss as compared with both the untreated mice and sham-infected mice (**Figures 1E–H**). This higher alveolar bone loss was demonstrated by the increased CEJ-ABC linear distance in the interdental area between the first and second molar (**Figure 1E**), the enhanced CEJ-ABC linear distance in the distal surface of the distal root of the first molar (**Figures 1F, G**), and the decreased percentage of remnant alveolar bone (**Figure 1H**) at both sides of the maxilla. To corroborate that this higher pathogenic potential was attributed to the *A. actinomycetemcomitans* serotype *b* instead of only one strain belonging to this serotype, the alveolar bone resorption was also analyzed in mice infected with another serotype *b* strain, the reference strain *A. actinomycetemcomitans* ATCC 23718. Similar levels of alveolar bone loss were detected in mice infected with the ATCC 23718 or VT1169 strains, determined as the CEJ-ABC linear distance in the interdental area (**Figure 1I**), CEJ-ABC linear distance in the distal root of the first molar (**Figures 1J, K**), and percentage of remnant alveolar bone (**Figure 1L**). When the *A. actinomycetemcomitans rmlC* mutant strain was used to generate the periodontal infections, significantly lower alveolar bone resorption was observed as compared with the periodontal infections induced with the *A. actinomycetemcomitans* VT1169 wild-type strain (**Figures 1E, F**). In mice infected with the *rmlC* mutant strain, the quantitative analysis of the CEJ-ABC linear distance shown in **Figure 1F** revealed a significant reduction in the alveolar bone loss as compared with mice infected with the VT1169 strain, with an average ranging between 0.213 and 0.161 mm (**Figure 1G**). Besides, this reduction represented an average increase of 7.27% in the remnant alveolar bone in *rmlC* mutant strain-infected mice, as compared with the VT1169 strain-infected mice (**Figure 1H**). The recovering of the *rmlC* gene in the *rmlC*/*rmlC*$^+$ complemented strain of *A. actinomycetemcomitans* led to an increase in the alveolar bone loss in infected mice, reaching similar levels than those detected in the VT1169 strain-infected mice (**Figures 1E–H**). Collectively, these results demonstrated that the O-PS moiety component of the LPS is involved in the virulence of *A. actinomycetemcomitans* and particularly, in the tooth-supporting alveolar bone loss induced during experimental periodontitis.

The O-PS-Defective *A. actinomycetemcomitans* Serotype *b* Mutant Strain Induces Decreased Th1, Th17, and Th22-Related Immune Responses Than the Wild-Type Strain

The immunogenic potential of the O-PS was assessed by analyzing the pattern of cytokines expressed in the periodontal

lesions. For this, we determined the gene expression of the proinflammatory cytokines related with Th1 (*Ifng* and *Il1b*), Th17 (*Il6*, *Il23*, *and Il17*), Th22 (*Il22*), and T regulatory (*Il10*) lymphocyte responses in the palatal periodontal tissues by qRT-PCR (**Figure 2A**). Significantly, lower expression levels for *Ifng* and *Il1b* were detected in the periodontal lesions of mice infected with the *A. actinomycetemcomitans rmlC* mutant strain, as compared with those infected with the VT1169 wild-type strain (**Figure 2B**). Furthermore, when the *rmlC* mutant strain was used to induce the periodontal lesions, significantly lower expression levels of the Th17-related cytokines, *Il17* and *Il23*, were detected as compared with periodontal lesions induced with the VT1169 wild-type strain (**Figure 2C**). The *Il6* expression levels were also lower in the *rmlC* mutant strain-induced periodontal lesions as compared with the VT1169 strain-induced lesions; however, these differences were not statistically significant (**Figure 2C**). Similarly, the detected differences in the expression of the Th22-related cytokine *Il22* were not statistically significant (**Figure 2D**). As a complement of the analysis of the Th22-related immune response, the expression levels of *Ahr*, transcription factor master-switch gene associated with the Th22 lymphocyte differentiation and function, were also quantified. In mice infected with the *rmlC* mutant strain, the expression levels for *Ahr* were significantly lower than those detected in mice infected with the VT1169 wild-type strain (**Figure 2D**). No significant differences were detected in the transcript levels of the immunoregulatory cytokine *Il10* among the studied groups; however, there was a slight overexpression in the *rmlC*-induced periodontal lesions (**Figure 2E**). When the *rmlC/rmlC*$^{+}$ complemented strain was used to induce periodontal lesions, the expression levels for *Ifng*, *Il1b*, *Il6*, *Il10*, *Il22, Il23*, and *Ahr* reached similar levels to those detected in periodontal lesions induced with the VT1169 wild-type strain, except for *Il17*, whose expression levels remained lower (**Figures 2B–E**). Overall, these data demonstrated that the O-PS moiety of the LPS is implied in the immunogenicity of *A. actinomycetemcomitans*. In particular, the O-PS-defective mutant strain induced lesser Th1, Th17, and Th22-patterns of immune response in periodontal lesions than the *A. actinomycetemcomitans* wild-type strain, and these reduced levels are likely to be involved in the decreased alveolar bone resorption observed during the induced periodontitis.

The O-PS-Defective *A. actinomycetemcomitans* Serotype *b* Mutant Strain Induces Less Expression of RANKL and TLR-4 Than the Wild-Type Strain

To confirm the association between the periodontal immune response and the observed changes in alveolar bone loss induced by the O-PS-defective *A. actinomycetemcomitans* mutant strain, we also determined the gene expression of the pro-osteolytic factor RANKL (*Tnfsf11*), the key factor involved the osteoclast differentiation and activation. In periodontal lesions induced with the *A. actinomycetemcomitans rmlC* mutant strain, the

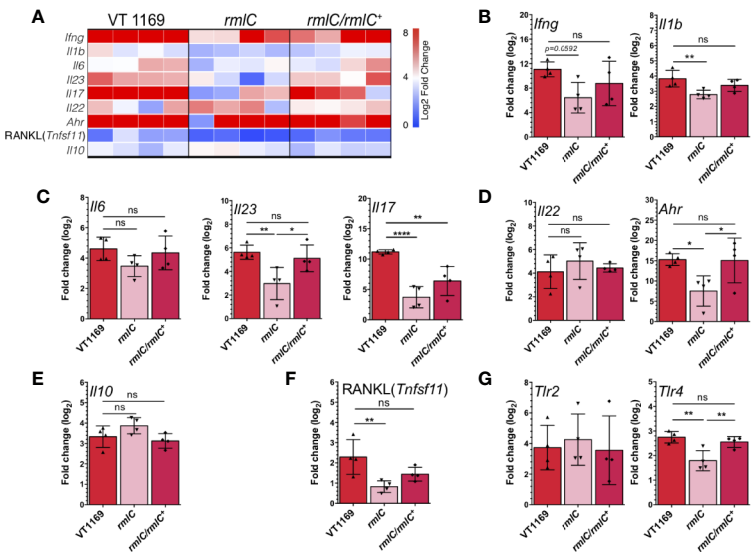

FIGURE 2 | Th1/Th17/Th22-related cytokines, RANKL, and TLR expression within the periodontal tissues induced by the different strains of *A. actinomycetemcomitans*. **(A)** Heatmap of mRNA expression levels for Th1, Th17, Th22, and T regulatory-related cytokines and RANKL quantified by qRT-PCR. Bar plots showing relative expression of **(B)** Th1-related *Ifng* and *Il1b*, **(C)** Th17-related *Il6*, *Il17*, and *Il23*, **(D)** Th22-related *Il22* and *Ahr*, **(E)** T regulatory-related *Il10*, **(F)** RANKL (*Tnfsf11*), and **(G)** *Tlr2* and *Tlr4* mRNAs analyses in periodontal lesions infected with the *A. actinomycetemcomitans* VT1169, *rmlC*, or *rmlC/rmlC*$^{+}$ strains (n=4). The data were pooled from three independent experiments. For relative expression, the mRNA expression in untreated (UT) mice was considered as 0, as a reference for fold-change in expression. Mean ± SD, one-way ANOVA and Tukey post-hoc test, *p < 0.05, **p < 0.01, ****p < 0.0001, ns, non-significant. Error bars represent SEM in all panels. *Ahr*, aryl hydrocarbon receptor; *Il*, interleukin; *Ifng*, interferon-gamma; *Tlr*, toll-like receptor; RANKL (*Tnfsf11*), receptor activator of nuclear-factor κB ligand.

expression levels for RANKL (*Tnfsf11*) were significantly lower as compared with those induced with the VT1169 wild-type strain (**Figure 2F**). When the *rmlC/rmlC*$^+$ complemented strain was used for the periodontitis induction, the transcript levels of *Tnfsf11* did not reach the levels expressed in the VT1169 wild-type strain-induced periodontal lesions; however, these differences were not statistically significant (**Figure 2F**). In order to get insights into the potential pathways involved in the O-PS recognition, we also quantified the expression levels of two Toll-like receptors related to LPS signaling, TLR-2 and TLR-4. In the periodontal lesions induced with the *A. actinomycetemcomitans rmlC* mutant strain, significantly lower transcript levels of *Tlr4* were detected, as compared with those induced with the VT1169 wild-type strain (**Figure 2G**). No differences were detected for the *Tlr2* transcript levels. When the *rmlC/rmlC*$^+$ complemented strain was used for the periodontitis induction, the expression of *Tlr4* reached similar levels to the ones detected in the VT1169 wild-type strain-induced periodontitis lesions (**Figure 2G**). This result suggests that the effect in the *Tlr4* expression could be attributed to the absence of O-PS, due to deletion in *rmlC* gene.

The O-PS-Defective *A. actinomycetemcomitans* Serotype *b* Mutant Strain Induces Less Detection of T-bet$^+$, RORγt$^+$, and AhR$^+$ T Lymphocytes Than the Wild-Type Strain in the Periodontal Lesions

Since our data showed the decreased expression levels of the Th1, Th17, and Th22-related cytokines in the periodontal lesions induced with the *A. actinomycetemcomitans* serotype *b* strain lacking the O-PS domain, we proceeded to determine the frequency and absolute number of the T helper lymphocytes infiltrating these periodontal lesions (**Figure 3A**). Flow cytometry analysis revealed a higher frequency and absolute number of Th1 (CD45$^+$CD3$^+$CD4$^+$T-bet$^+$) (**Figures 3B, C**), Th17 (CD45$^+$CD3$^+$CD4$^+$RORγt$^+$) (**Figures 3D, E**), and Th22 (CD45$^+$CD3$^+$CD4$^+$AhR$^+$) (**Figures 3F, G**) lymphocytes in periodontal lesions of mice infected with the VT1169 wild-type strain compared with the periodontal tissues of untreated control mice. No differences were found in T-cell viability among the different experimental conditions, with an average of 95.81% viability of cells obtained from infected periodontal lesions (**Supplementary Figure S3**). Within periodontal lesions induced with the *rmlC* mutant strain, the T-bet$^+$ T lymphocyte detection was significantly lower in frequency and absolute number, as compared with the VT1169 wild-type strain-induced periodontal lesions (**Figures 3B–C**). When the periodontal lesions were induced with the *rmlC/rmlC*$^+$ complemented strain, the frequency and absolute number of T-bet$^+$ T lymphocytes increased, reaching levels similar to those detected in the periodontal lesions induced with the VT1169 wild-type strain (**Figures 3B–C**). Similarly, the absolute number of infiltrating RORγt$^+$ T lymphocytes was significantly decreased by almost half, within the *rmlC* mutant strain-induced periodontal lesions as compared with the VT1169 wild-type strain-induced periodontal lesions (**Figures 3D–E**). No differences were detected in the frequency of RORγt$^+$ T lymphocytes between the periodontal lesions induced with the *rmlC* mutant strain and *rmlC/rmlC*$^+$ complemented strain; however, when the absolute cell number was analyzed, a no-significant increment in the RORγt$^+$ T lymphocyte detection was observed in the *rmlC/rmlC*$^+$ complemented strain-induced periodontal lesions (**Figure 3E**). Since it was recently suggested that Th22 lymphocytes could be associated with the RANKL-mediated alveolar bone loss in a mice model of *A. actinomycetemcomitans*-induced experimental periodontitis (32), we also compared the infiltration of AhR$^+$ T lymphocytes within the periodontal lesions. No significant differences were detected in the frequency of AhR$^+$ T lymphocytes between the *rmlC* mutant strain-induced and VT1169 wild-type strain-induced periodontal lesions (**Figures 3F, G**); however, a significantly lower AhR$^+$ T lymphocyte number was quantified in periodontal lesions induced with the *rmlC* mutant strain as compared with those induced with the VT1169 wild-type strain (**Figures 3F, G**). When the *rmlC/rmlC*$^+$ complemented strain was used to induce periodontal lesions, the frequency and absolute number of Th22 reached similar levels to the ones detected in periodontal lesions induced with the VT1169 wild-type strain (**Figures 3F, G**). Taken together, these results allow us to suggest that the absence of the O-PS domain in the LPS from *A. actinomycetemcomitans* serotype *b* significantly affects its capacity to induce Th1, Th17, and Th22 lymphocyte infiltration in experimental periodontal lesions.

We next assessed whether the variations observed in the periodontal lesions are reflected in the frequency and absolute number of Th1, Th17, and Th22 lymphocytes within the oral-draining lymph nodes. We observed discrepancies in the absolute number of total T helper cells between the periodontal lesions and cervical lymph nodes (**Figure 4A**). To further determine whether the specific T helper subsets showed similar variations to those found in periodontal lesions, the frequency and absolute number of T-bet$^+$, RORγt$^+$, and AhR$^+$ T lymphocytes were determined in the cervical lymph nodes. Similarly, neither the frequency nor the absolute cell number of T-bet$^+$, RORγt$^+$, or AhR$^+$ T lymphocytes significantly varied among the different experimental conditions (**Figures 4B–G**). Therefore, the decreased Th1, Th17, and Th22 lymphocyte detection in the periodontal lesions induced with the *A. actinomycetemcomitans rmlC* mutant strain was not reflected in changes in T helper lymphocyte detection in the cervical lymph nodes that drain these periodontal lesions.

The O-PS-Defective *A. actinomycetemcomitans* Serotype *b* Mutant Strain Induces Less Expression of CD40 and CD80 in Dendritic Cells and B Lymphocytes Than the Wild-Type Strain

Since T helper cell polarization and expansion requires not only the presence of bacterial antigen and cytokines, but also co-stimulatory molecules expressed by pathogen-experienced antigen-presenting cells (APCs), including dendritic cells (DCs), macrophages, and B

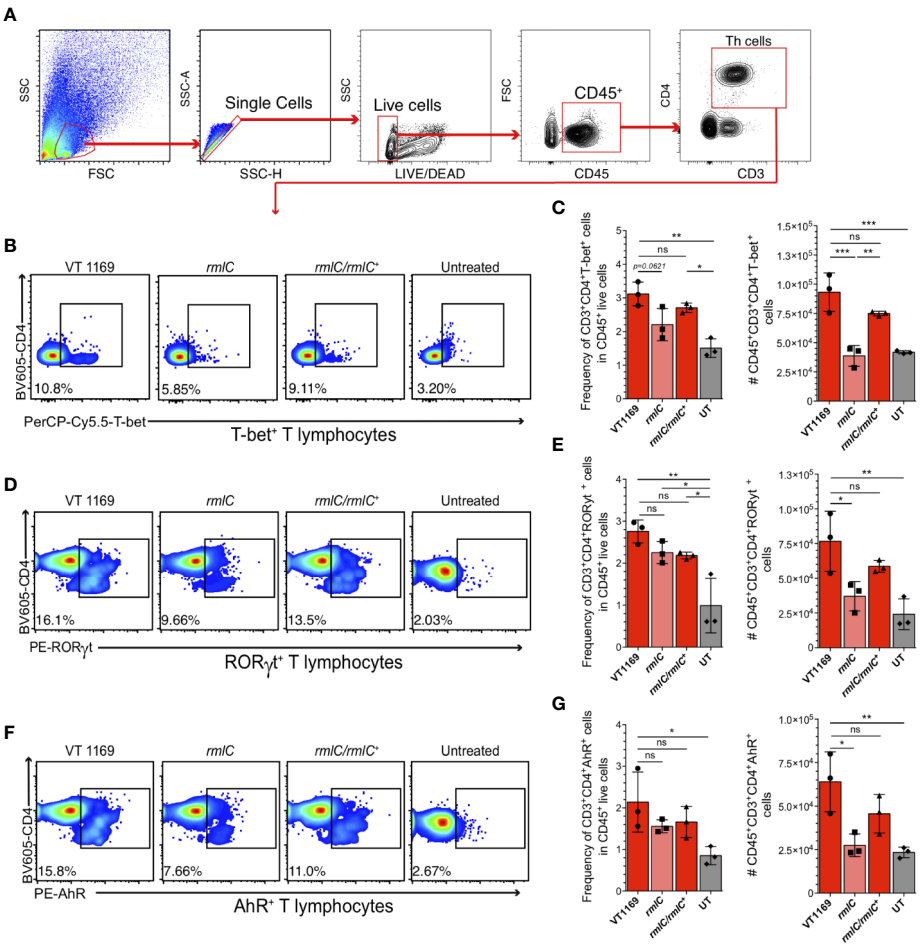

FIGURE 3 | T-bet$^+$, RORγt$^+$, and AhR$^+$ T lymphocytes detection within the periodontal tissues induced by the different strains of *A. actinomycetemcomitans*. Cells obtained from periodontal tissues were analyzed by flow cytometry. **(A)** Gating strategy used for lineage determination of single/live/CD45$^+$CD3$^+$CD4$^+$ T lymphocytes. Flow cytometric quantification of **(B**, **C)** CD45$^+$CD3$^+$CD4$^+$T-bet$^+$ Th1 lymphocytes, **(D**, **E)** CD45$^+$CD3$^+$CD4$^+$RORγt$^+$ Th17 lymphocytes, and **(F**, **G)** CD45$^+$CD3$^+$CD4$^+$AhR$^+$ Th22 lymphocytes in periodontal lesions upon infection with the *A. actinomycetemcomitans* VT1169, *rmlC*, or *rmlC/rmlC*$^+$ strains, or the untreated (UT) controls. Plots are representative of three independent experiments (n=3). Mean ± SD, one-way ANOVA and Holm-Sidak post-hoc test, *p < 0.05, **p < 0.01, ***p < 0.001, ns, non-significant. Error bars represent SEM in all panels.

lymphocytes (35), we examined the surface expression of the co-stimulatory molecules CD40 and CD80 in mice APCs. First, to determine the frequency of APCs present in the mice periodontal tissues at steady state, a flow cytometry characterization of the immune cell network followed by a dimensionality reduction with the T-distributed stochastic neighbor embedding (tSNE) algorithm was carried out (**Figure 5A**). Using a panel that allowed us to detect B lymphocytes (CD45$^+$CD19$^+$MHC-II^{hi}), T lymphocytes (CD45$^+$CD3$^+$CD90$^+$), innate lymphoid cells (CD45$^+$CD90$^+$CD3$^-$), macrophages (CD45$^+$CD64$^+$CD11b$^+$), DCs (CD45$^+$CD11c$^+$MHC-II^{hi}), neutrophils (CD45$^+$Ly6G$^+$Ly6C^{mid}), and monocytes (CD45$^+$Ly6C$^+$Ly6G$^-$) (**Supplementary Figure S4**), we determined that the main APCs present in the periodontal tissues were B lymphocytes (75.0%), DCs (2.56%), and macrophages (1.6%) (**Figure 5B**). These results were crucial to design a proper experimental set up to analyze the changes in APCs in periodontal lesions, while trying to emulate, as much as possible, the mice gingival barrier context *in vitro*.

Among the co-stimulatory molecules, CD40 and CD80 are critical signals expressed by APCs for optimal activation of T lymphocytes (36). Since we determined that B lymphocytes, DCs, and macrophages are the main APCs present in the mice periodontal tissues, we evaluated whether the lack of O-PS in the *A. actinomycetemcomitans* serotype *b* strain alters the CD40 and CD80 surface expression on bacteria-challenged splenocytes-derived APCs. Splenocytes were used based on the similarities in APC proportions between the spleen and periodontal tissues in mice (37) (**Supplementary Figure S5**). When B lymphocytes were challenged with the VT1169 wild-type strain, the expression levels of CD40 and CD80 augmented significantly, reaching similar levels to those expressed in B lymphocytes stimulated with *E. coli*-derived

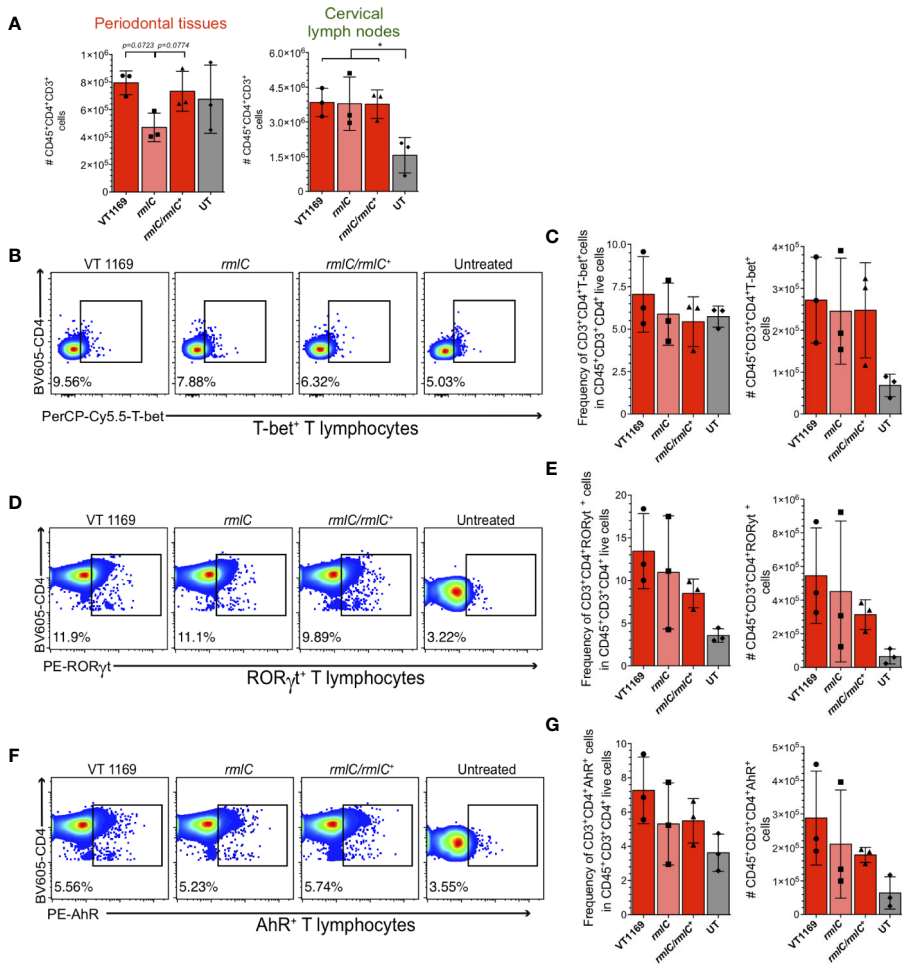

FIGURE 4 | T-bet$^+$, RORγt$^+$, and AhR$^+$ T lymphocytes detection within the cervical lymph nodes induced by the different strains of *A. actinomycetemcomitans*. Cells obtained from cervical lymph nodes were analyzed by flow cytometry. **(A)** Comparison of the number of single/live/CD45$^+$CD3$^+$CD4$^+$ T lymphocytes between periodontal tissues and cervical lymph nodes. Flow cytometric quantification of **(B, C)** CD45$^+$CD3$^+$CD4$^+$T-bet$^+$ Th1 lymphocytes, **(D, E)** CD45$^+$CD3$^+$CD4$^+$RORγt$^+$ Th17 lymphocytes, and **(F, G)** CD45$^+$CD3$^+$CD4$^+$AhR$^+$ Th22 lymphocytes in periodontal lesions upon infection with the *A. actinomycetemcomitans* VT1169, *rmlC*, or *rmlC/rmlC*$^+$ strains, or the untreated (UT) controls. Data were pooled from three independent experiments (n=3). Mean ± SD, one-way ANOVA and Holm-Sidak post-hoc test, *p < 0.05. Error bars represent SEM in all panels.

LPS, used as positive control (**Figures 5C, F**). The exposure of B lymphocytes to the *rmlC* or *waaL* mutant strains led to lower CD40 and CD80 expression levels to those detected in the VT1169 wild-type strain-challenged cells (**Figures 5C, F**). When the B lymphocytes were challenged with the *rmlC/rmlC*$^+$ or *waaL/waaL*$^+$ complemented strains, the expression levels of CD40 and CD80 augmented, reaching similar levels to those detected in B lymphocytes challenged with the VT1169 wild-type strain (**Figures 5C, F**). Similarly, the VT1169 wild-type strain-challenged DCs significantly increased the expression of CD40 and CD80, reaching similar levels to those expressed in DC stimulated with *E. coli*-derived LPS (**Figures 5D, G**). When the DCs were challenged with the *rmlC* mutant strain, only the expression of CD80 was significantly decreased as compared with VT1169 wild-type strain-challenged DCs (**Figures 5D, G**). In the DCs stimulated with the *waaL* mutant strain, the expression of CD80 was lower as compared with the DCs stimulated with the VT1169 wild-type strain; however, these differences were not statistically significant (**Figures 5D, G**). The stimulation of DCs with the *rmlC/rmlC*$^+$ or *waaL/waaL*$^+$ complemented strains significantly increased the expression of CD80, reaching similar levels to those detected in VT1169 wild-type strain-challenged DCs (**Figure 5D**). Although macrophages increased the surface expression of CD40 and CD80 after *E. coli*-derived LPS or *A. actinomycetemcomitans* strains exposure, no significant differences on CD40 and CD80 expression were found among the different bacteria-stimulated conditions (**Figure 5E**). Importantly, no significant differences in cell viability were detected in splenocytes challenged with the aforementioned strains (**Supplementary Figure S6**).

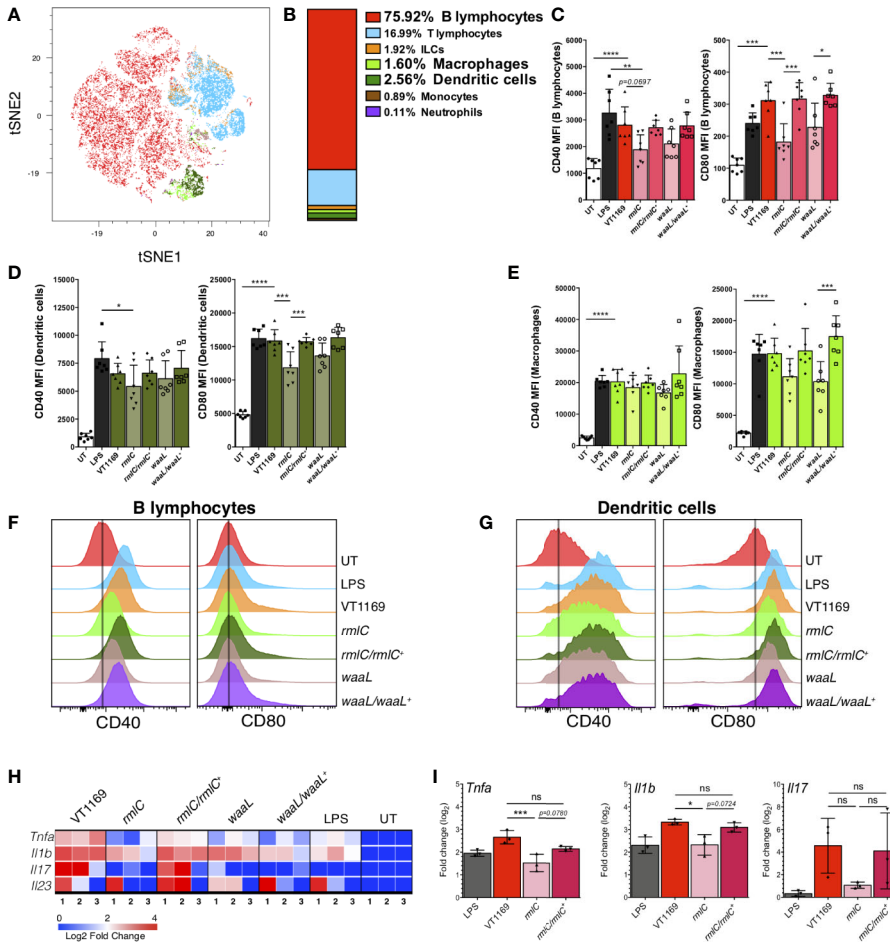

FIGURE 5 | B-lymphocyte, dendritic cell, and splenocyte responses against the different strains of *A. actinomycetemcomitans*. **(A)** tSNE map of 11-parameter flow cytometry data from untreated (UT) mice, color-coded as follows: B-cells in red, T-cells in cyan, innate lymphoid cells (ILCs) in orange, macrophages in light green, dendritic cells in dark green, monocytes in brown, and neutrophils in purple. **(B)** Percentage of each cell population described in **(A)**. Data pooled from two independent experiments (n=4). The MFI levels of the co-stimulatory molecules CD40 and CD80 are shown in **(C)** for B lymphocytes, **(D)** for dendritic cells, and **(E)** macrophages stimulated 20 h with the *A. actinomycetemcomitans* VT1169, *rmlC*, *rmlC/rmlC*$^{+}$, *waaL*, or *waaL/waaL*$^{+}$ strains, and the *E. coli*-derived LPS-stimulated and UT cells, used as controls. The histograms representing the MFI levels of the co-stimulatory molecules CD40 and CD80 are shown in **(F)** for B lymphocytes and **(G)** for dendritic cells, under the same conditions experimental described in **(C-E)**. Data pooled from three different experiments (n=7). **(H)** Heatmap and **(I)** bar plots of qRT-PCR analysis of *Tnfa*, *Il1b*, *Il17*, and *Il23* transcripts in total splenocytes stimulated 8 h with the *A. actinomycetemcomitans* VT1169, *rmlC*, *rmlC/rmlC*$^{+}$, *waaL*, or *waaL/waaL*$^{+}$ strains, and the *E. coli*-derived LPS-stimulated and UT cells, used as controls (n=3). Data pooled from two independent experiments. Mean ± SD, one-way ANOVA and Tukey post-hoc test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Error bars represent SEM in all panels. *Il*, interleukin; LPS, *E. coli*-derived lipopolysaccharide; *Tlr*, toll-like receptor; *Tnfa*, tumor necrosis factor-alpha.

The O-PS-Defective *A. actinomycetemcomitans* Serotype *b* Mutant Strain Induces Less Expression of *Tnfa* and *Il1b* in Splenocytes Than the Wild-Type Strain

Besides, the expression levels of the NF-κB target genes *Tnfa* and *Il1b*, as well as *Il17 and Il23*, were measured in total splenocytes stimulated with the aforementioned *A. actinomycetemcomitans* strains after 8 h, showing that the absence of the O-PS domain impaired the transcript levels of those proinflammatory cytokines (**Figure 5H**). In particular, when splenocytes were exposed to the *rmlC* mutant strain, the expression levels of *Tnfa*, *Il1b*, and *Il17* were lower to those detected on the VT1169 wild-type strain-challenged splenocytes; however, the compared levels of *Il17* showed no statistically significant differences (**Figure 5I**). When the splenocytes were challenged with the *rmlC/rmlC*$^{+}$ complemented strain, the expression levels of *Tnfa*, *Il1b*, and *Il17* augmented, reaching similar levels to those detected in splenocytes challenged with the VT1169 wild-type strain (**Figure 5I**). Collectively, these results demonstrate that the *A. actinomycetemcomitans* serotype *b* O-PS domain is key to trigger the gene expression of the proinflammatory cytokines *Tnfa* and *Il1b* on immune cells, and it is highly involved in the upregulation of the surface co-stimulatory molecules CD40 and CD80 on B lymphocytes and DCs.

DISCUSSION

A. actinomycetemcomitans strains belonging to the serotype *b* show higher *in vitro* and *in vivo* immunogenicity and are more frequently isolated from the periodontal lesions of periodontitis-affected patients when compared with the other serotypes. Within this serotype *b* cluster, most studies have focused on the pathogenic mechanisms associated with the leukotoxin, while the virulence and immunogenic potential of the conserved O-PS component of its LPS have been largely understudied. In the current study, we investigated the virulence and immunogenicity of a transposon-derived *A. actinomycetemcomitans* serotype *b* mutant strain, characterized by the deletion of the *rmlC* gene and the impaired expression of its O-PS. This O-PS-defective *A. actinomycetemcomitans* strain provoked lower alveolar bone resorption, decreased infiltration of Th1, Th17, and Th22 lymphocytes, and downregulated expression of *Ahr*, *Il1b*, *Il17*, *Il23, Tlr4*, and RANKL (*Tnfsf11*) in experimental periodontal lesions, as compared with the wild-type strain. These variations were focalized in the periodontal tissues, without significant disturbance in the T helper subsets within the cervical lymph nodes. During the characterization of the gingival immune compartment, we also determined that the most frequent periodontal APCs were B lymphocytes and DCs. In these cells, O-PS-defective *A. actinomycetemcomitans* strain challenge triggered a lower expression of the co-stimulatory molecules CD40 and CD80, as well as lower expression of *Tnfa* and *Il1b*, as compared with the wild-type strain, suggesting that the lack of this bacterial moiety could affect their capacity to prime T lymphocytes towards the pro-osteolytic phenotypes.

Consistent with previous reports (18, 32, 38, 39), our results demonstrated that the oral inoculation of the serotype *b* VT1169 wild-type strain was capable of inducing bone loss. Conversely, when the periodontal lesions were induced with the *rmlC* mutant strain lacking the O-PS moiety, the mice developed less severe alveolar bone loss. Based on this observation, we propose that the O-PS domain exerts a critical role in the virulence of the serotype *b* cluster. This may be due to changes in the periodontal immune cell network mediating the activation of tooth-supporting bone resorption in response to structural variations in bacterial LPS, as it was previously reported with the purified LPS of *A. actinomycetemcomitans* serotype *b* (40–42).

The immunodominant epitope of the LPS from the *A. actinomycetemcomitans* serotype *b* resides in its O-PS domain (33, 34). We have previously reported that serotype *b* induces higher alveolar bone resorption than the other serotypes, and this increased bone loss was associated with the Th1 and Th17-lymphocyte activity in periodontal lesions (18). Besides, the serotype *b* has demonstrated increased immunogenicity *in vitro*, as it exerts a key role in phagocytosis resistance by human neutrophils (43), promotes the formation of osteoclast-like cells from bone marrow cells (44), induces the release of monocyte and T lymphocyte chemotactic factors, such as CCL2 and IL-8, and the secretion of IL-1 in challenged murine macrophages (45), triggers the expression of proinflammatory cytokines in stimulated monocytes (46) and dendritic cells (21), and induces the production of RANKL in T lymphocytes (47). All of these capacities of *A. actinomycetemcomitans* serotype *b* have been attributed to the O-PS structure of its LPS. In the present study, when periodontal lesions were induced with *A. actinomycetemcomitans* lacking the O-PS domain, we observed a significant reduction in the transcript levels of the cytokines IL-1β, IL-17, and IL-23, the Th22-associated transcription factor AhR, and the osteoclastogenic mediator RANKL. In line with the data obtained from the *in vivo* experiments, splenocytes challenged with the *rmlC* mutant strain expressed significantly less transcript levels of TNFα and IL1β, ratifying the importance of the O-PS fraction in *A. actinomycetemcomitans* immunogenicity.

The decreased transcript levels of IL-1β induced by the *rmlC* mutant could be involved in its diminished virulence, as previous works have shown that IL-1β inhibition reduced bone loss in experimental periodontitis (48, 49). Nonetheless, alveolar bone resorption during periodontitis is mainly mediated by the Th17-type of immune response, which is central in the periodontal osteo-immune network crosstalk (50). In infected mice, the absence of O-PS also diminished the expression levels of the Th17-related cytokines IL-23 and IL-17. IL-23 is directly involved in the Th17 osteolytic phenotype maintenance and Th22 lymphocyte differentiation and could be secreted by APCs, contributing to the polarization of *naïve* T cells and the perpetuity of lymphocytic pathogenic activity. Further, IL-17, the signature Th17-type cytokine, is precisely involved in immune-mediated alveolar bone resorption by directly promoting osteoclastogenesis and inducing RANKL expression on periodontal osteoblasts and fibroblasts (51). In this context, we also found that the inoculation of the *rmlC* mutant strain ameliorated the expression of RANKL in periodontal lesions. Potentially, this perturbation in the cytokine milieu could be due to changes in a plethora of cells that have the ability to recognize the O-PS, including immune and tissue-resident cells. However, it is well known that the key players in the response against immunostimulatory oral pathogens mostly belong to the immune cell compartment. Herein, based on the significant perturbations observed in the periodontal Th1, Th17, and Th22-associated proinflammatory and pro-osteolytic cytokine milieu in the periodontal tissues, we can conclude that the O-PS from the *A. actinomycetemcomitans* serotype *b* is key in the occurrence of inflammatory events that lead to alveolar bone destruction. Whether these perturbations are mainly mediated by immune cells is a matter of future research.

To dissect the influence of the O-PS in the gingival immune cell compartment, we analyzed the changes in the frequency and absolute number of T helper subsets in the periodontal tissues, focusing on the Th1 and Th17 proinflammatory and osteoclastogenic subsets. Our previous works have revealed that *A. actinomycetemcomitans* serotype *b* induces greater infiltration of Th1 and Th17 cells within experimental periodontal lesions, as compared with serotypes *a* or *c* (18). Besides, the serotype *b*-induced alveolar bone loss was also associated with increased infiltration of $IL22^{+}AhR^{+}$ Th22 lymphocytes within periodontal lesions (32). Indeed, IL-22 levels correlated with RANKL expression in periodontal lesions, suggesting a role of Th22

lymphocytes in the RANKL-mediated bone loss (32). In the present study, we found that the lack of O-PS in the *rmlC* serotype *b* mutant strain caused less infiltration of Th1, Th17, and Th22 lymphocytes in periodontal lesions as compared with the wild-type strain. In this context, Th1 lymphocytes are the main producers of the proinflammatory cytokines IL-1β, IL-12, IFN-γ, and TNF-α, with a transcriptional program controlled by the transcription factor T-bet. In fact, *A. actinomycetemcomitans*-induced periodontitis in IFN-γ-knockout mice resulted in decreased alveolar bone resorption accompanied by an impaired host defense against microbial dissemination followed by mice death, showing the key role of the Th1-related type-I IFN signaling in the local immune-mediated pathological bone loss and host protective response against *A. actinomycetemcomitans* (52). Thus, the diminished bone loss observed in our experimental approach could be in part due to impaired Th1 differentiation and/or infiltration into periodontal lesions. Accordingly, the ablation of CCL3/CCR1/CCR5 Th1-associated chemotactic axis in mice challenged with *A. actinomycetemcomitans* protected them from alveolar bone loss due to decreased Th1 lymphocyte infiltration (53). Even so, Th17 lymphocytes are considered the main bridge between immune response and bone metabolism due to their ability to directly express RANKL and induce RANKL expression in periodontal fibroblasts through IL-17 production (51). In turn, Th22 lymphocytes have also been described as an osteoclastogenic T helper subset (54). Similar to IL-17, IL-22 can directly promote osteoclast differentiation (54) and induce RANKL expression in periodontal ligament fibroblasts (55). Our results showed that components of the periodontal immune cell compartment are able to detect changes in the structure of O-PS from the *A. actinomycetemcomitans* LPS, which resulted in the modification of the local immune response mediated by the Th1, Th17, and Th22 lymphocytes. We cannot totally discard that other immune players could exert a role in mediating alveolar bone loss in response to variations in the presence of O-PS, including other T lymphocyte subsets such as Th2, T regulatory, or γδ-T cells, among others. Despite the fact that, to date, the knowledge of the oral immune landscape is still limited, most of the studies point to the protagonism of these three T helper subsets, particularly to the Th1 and Th17 lymphocytes, during the immuno-mediated alveolar bone resorption. In this regard, future studies are needed to dissect in deep all the mechanisms and immune cells participating in the variability of bone loss observed when the O-PS moiety of *A. actinomycetemcomitans* was absent.

Interestingly, the variation pattern observed at the periodontal level was not mirrored at the cervical lymph nodes, as could be expected. Whether these differences are an effect of variations in the leukocyte trafficking, tissular disturbances in chemotactic signals, or due to local changes in the orchestration of T helper cell differentiation at tertiary lymphoid structures is something that, unfortunately, was not addressed in this study and it needs to be further investigated. Overall, together with the fact that the *rmlC* mutant strain causes a reduced proinflammatory and osteoclastogenic cytokine network in the periodontal lesions, our data suggest that the lack of O-PS domain may interfere with the local mechanisms of inflammation and alveolar bone resorption mediated by Th1, Th17, and Th22 lymphocytes. These results, however, should be interpreted with caution, as the approach used here to detect T helper subsets was based only on their master transcription factors. It is known that $Foxp3^+$ regulatory T cells could co-express RORγt and maintain or even enhance their suppressive capacity (56). Based on that, we cannot rule out the possibility that $ROR\gamma t^+$ T cells are also displaying regulatory functions in our system. To solve this, future studies dissecting the heterogeneity and functional plasticity of the analyzed T helper subsets in our model of experimental periodontitis are needed.

The activation of the adaptive immunity depends on co-stimulatory surface molecules expressed by APCs that regulate the magnitude and quality of the T helper cell response, including CD40, CD80, and CD86. Indeed, the aforementioned Th1, Th17, and Th22 lymphocytes require these signals to orchestrate an appropriate adaptive immune response. The recognition of peptide/MHC II complexes on DCs by $CD4^+$ T lymphocytes upregulates the CD40 ligand (CD40L), and the CD40/CD40L interactions, in turn, 'license' DCs for T-cell priming by overexpressing CD80 and CD86 (57). We found that the absence of O-PS affected the surface expression of the co-stimulatory molecules CD40 and CD80 in B lymphocytes and DCs, thus potentially disturbing the priming capacity of these APCs. This could explain, at least in part, the variation in frequency and number of the different T helper subsets that we detected in the periodontal tissues. Additionally, our results revealed that DCs exert higher surface expression of CD40 and CD80, as compared with B lymphocytes, emphasizing their role as professional APCs. Similar results were found when human DCs were exposed to mutant variants of *Neisseria meningitidis* LPS lacking O-PS, which exhibited a decreased surface expression of CD80 and CD86 (58). Otherwise, DCs that lack CD40 and CD80 have enhanced ability to induce T regulatory cells, while weakly direct other T helper cell subsets differentiation (59). Furthermore, the higher expression of CD40 on licensed DCs may favor the Th1 differentiation (60). Based on our results, we could establish that APCs, and particularly DCs and B lymphocytes, may be able to sense the presence of O-PS in the *A. actinomycetemcomitans* LPS structure, affecting their priming capacity and the quality of the T-cell mediated immune response. Further studies are needed with a focus on the pattern recognition receptor (PRR) signaling to determine the pathways involved in the recognition of O-PS by periodontal APCs. In this context, the immune response against *P. gingivalis* LPS is unusual due to its unique and heterogenous lipid A structure, being considered an agonist for TLR-2 as well as an agonist or antagonist for TLR-4 (61, 62). In fact, *P. gingivalis* LPS or its purified lipid A moiety were able to activate TLR4-deficient macrophages obtained from C3H/HeJ mice, demonstrating their signaling *via* TLR-2 (63, 64). However, the lipid A moiety is highly conserved in *A. actinomycetemcomitans* and taxonomically close species, such as *Aggregatibacter aphrophilus* (65, 66), highlighting

the potential role of the O-PS in the variability of *A. actinomycetemcomitans* immunogenicity. Thus, the understanding of the variability in the *A. actinomycetemcomitans* O-PS/PRR signaling could provide evidence to further elucidate potential mechanisms underlying our findings.

The *rmlC* gene has also been involved in the mediation of leukotoxin secretion *via* the TolC-dependent type I secretion system (T1SS) (27), and in the collagen adhesion, through glycosylation of the extracellular matrix protein adhesin A (EmaA) (26). Hence, we cannot rule out that our results are, at least in part, also associated with changes in these virulence mechanisms potentially affected by the deletion of the *rmlC* gene in *A. actinomycetemcomitans*. However, the leukotoxin is an exotoxin associated with immune evasion through the induction of lymphocytes apoptosis (67); thus, increased T-cell survival and consequently, enhanced local T-cell mediated immune response would be expected with a reduction in the leukotoxin production, instead of the immune response ablation observed in our findings. Besides, no differences were found in periodontal T helper cell survival when challenged with the wild-type, mutant, and complemented strains of *A. actinomycetemcomitans* (**Supplementary Figure S3**). Since leukotoxin is a primate-specific exotoxin (23) and we used a murine model of periodontitis and mouse-derived splenocytes, we could speculate that the effects on leukotoxin secretion related to the *rmlC* gene must be exiguous in the immune perturbations observed in our experimental approaches. On the other hand, since the periodontal lesions were induced by direct bacterial microinjections into periodontal tissues, avoiding interactions with commensal microbiota and gingival keratinocytes, the role of adhesins needed for bacterial colonization should not be playing a major role in the observed periodontal immune changes.

In the current study, *A. actinomycetemcomitans* strains belonging to the serotype *b* were used in all experiments due to their higher immunogenic potential as compared with the other serotypes. Indeed, *A. actinomycetemcomitans* strains belonging to the serotype *b* have demonstrated a higher capacity to trigger *Ifng*, *Tnfa*, *Il1b*, *Il6*, *Il12*, and *Il23* expression in human monocyte-derived DCs as compared with *A. actinomycetemcomitans* strains belonging to the other serotypes (21). Besides, different *in vitro* and *in vivo* studies have ratified the higher immunogenicity and virulence of serotype *b* ATCC 43718 strain as compared with serotypes *a* or *c* (18–21, 29, 47). Interestingly, the *A. actinomycetemcomitans* strains ATCC 29522, ATCC 43718, and ATCC 29524, all of them belonging to serotype *b*, induced similar overexpression of *Ifng*, *Tnfa*, *Il1b*, *Il6*, *Il12*, and *Il23*, demonstrating that the higher immunogenic potential attributed to the serotype *b* is conserved (21). Even so, considering the genetic diversity of *A. actinomycetemcomitans*, more studies are needed to ratify the role of the O-PS from *A. actinomycetemcomitans* serotype *b*, for example, by using different strains belonging to this serotype. Overall, our findings contribute to a better understanding of a virulence factor that could be involved in the higher immunogenicity and virulence of *A. actinomycetemcomitans* serotype *b* strains. Accordingly, previous *in vitro* data already suggested higher immunogenicity of serotype *b* O-PS as compared with serotypes *a* or *c* O-PS. Indeed, murine macrophages challenged with O-PS purified from *s*erotype *b* produced higher levels of IL-1 than the same cells challenged with O-PS purified serotypes *a* or *c* strains (45). Despite this, to gain a deep understanding of how the O-PS from serotypes *a*, *b*, and *c* contribute to the distinct immunogenicity and virulence of *A. actinomycetemcomitans*, future studies addressing their effects *in vivo* are needed, ideally using clinical isolates.

From a translational view, our findings contribute to a better understanding of the virulence factors involved in the immunostimulatory and pathogenic potential of *A. actinomycetemcomitans*. In particular, herein, we demonstrated the role of *A. actinomycetemcomitans* O-PS in the triggering of critical pathological mechanisms involved in the clinical presentation of periodontitis. Several epidemiological studies have clearly reported the direct association between *A. actinomycetemcomitans* strains belonging to the serotype *b* and severe forms of periodontitis (2, 15–17). Thus, the gained insights on the virulence mechanisms of this group of strains could help to increase the accuracy of diagnosis, treatment, and prognosis of periodontitis. Similarly, the better comprehension of the pathogenic mechanisms of *A. actinomycetemcomitans* involved in the pathogenesis of other systemic conditions, including rheumatoid arthritis (68) and atherosclerosis (69), could contribute to understanding how oral conditions could affect the systemic health, particularly due to the systemic dissemination of oral bacteria, such as *A. actinomycetemcomitans*. In this regard, special emphasis should be put on the development of precise microbiological diagnostic tools that could allow identifying the presence of highly virulent *A. actinomycetemcomitans* strains, thus better-determining risk profiles and personalized treatments of oral and oral-related systemic diseases.

Recent epidemiological studies have revealed that the prevalence of periodontitis is higher in males than females; thus, potential sex-related periodontitis susceptibility needs to be considered (70). Indeed, some evidence points to sex dimorphism related to sex steroids effects on the host's immune responses and the control of subgingival microbiota, being estrogen associated with a decreased susceptibility to periodontitis (71, 72). However, mice models of periodontitis have revealed an opposite trend, with higher susceptibility to alveolar bone loss and periodontal inflammation in female mice as compared with male mice (73). In the present study, only female mice were used, due to the higher increased periodontitis susceptibility could allow detecting small differences between the mice periodontal status. Nonetheless, more studies are needed to elucidate the potential role of sex-specific determinants on the capacity of *A. actinomycetemcomitans* O-PS to induce periodontitis.

To our knowledge, this is the first study reporting the role of O-Polysaccharide in the immunogenicity and virulence of *A. actinomycetemcomitans* serotype *b* in an *in vivo* model of periodontitis. Our results confirm that this bacterial moiety affects the maturation of APCs, the Th1, Th17, and Th22-pattern of the periodontal immune response, and the alveolar bone resorption during experimental periodontitis, playing a

significant role in the virulence and immunogenicity of this highly virulent periodontal bacteria.

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 animal study was reviewed and approved by Institutional Committee for Animal Care and Use from Universidad de Chile (Ethical permit #061601) and the Stockholm Regional Ethics Committee.

AUTHOR CONTRIBUTIONS

GM conceived the study, designed and performed most experiments, analyzed the data, and was involved in drafting the article. FC, EC, JA, and CT-A performed experiments. AH and EV designed experiments, analyzed the data, and critically evaluated and supplemented the article. RV conceived the study, designed experiments, analyzed the data, and prepared the article for submission. 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.2020.591240/full#supplementary-material

REFERENCES

1. Zambon JJ, Umemoto T, De Nardin E, Nakazawa F, Christersson LA, Genco RJ. *Actinobacillus actinomycetemcomitans* in the pathogenesis of human periodontal disease. *Adv Dent Res* (1988) 2(2):269–74. doi: 10.1177/08959374880020021101
2. Haubek D, Ennibi OK, Poulsen K, Vaeth M, Poulsen S, Kilian M. Risk of aggressive periodontitis in adolescent carriers of the JP2 clone of *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* in Morocco: a prospective longitudinal cohort study. *Lancet* (2008) 371(9608):237–42. doi: 10.1016/S0140-6736(08)60135-X
3. Fine DH, Markowitz K, Furgang D, Fairlie K, Ferrandiz J, Nasri C, et al. *Aggregatibacter actinomycetemcomitans* and its relationship to initiation of localized aggressive periodontitis: longitudinal cohort study of initially healthy adolescents. *J Clin Microbiol* (2007) 45(12):3859–69. doi: 10.1128/JCM.00653-07
4. Graves DT, Li J, Cochran DL. Inflammation and uncoupling as mechanisms of periodontal bone loss. *J Dent Res* (2011) 90(2):143–53. doi: 10.1177/0022034510385236
5. Takayanagi H. Inflammatory bone destruction and osteoimmunology. *J Periodontal Res* (2005) 40(4):287–93. doi: 10.1111/j.1600-0765.2005.00814.x
6. Hajishengallis G, Darveau RP, Curtis MA. The keystone-pathogen hypothesis. *Nat Rev Microbiol* (2012) 10(10):717–25. doi: 10.1038/nrmicro2873
7. Jiao Y, Hasegawa M, Inohara N. Emerging roles of immunostimulatory oral bacteria in periodontitis development. *Trends Microbiol* (2014) 22(3):157–63. doi: 10.1016/j.tim.2013.12.005
8. Darveau RP. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol* (2010) 8(7):481–90. doi: 10.1038/nrmicro2337
9. Asikainen S, Chen C. Oral ecology and person-to-person transmission of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *Periodontol 2000* (1999) 20:65–81. doi: 10.1111/j.1600-0757.1999.tb00158.x
10. Asikainen S, Chen C, Saarela M, Saxen L, Slots J. Clonal specificity of *Actinobacillus actinomycetemcomitans* in destructive periodontal disease. *Clin Infect Dis* (1997) 25 Suppl 2:S227–9. doi: 10.1086/516211
11. Chen C, Wang T, Chen W. Occurrence of *Aggregatibacter actinomycetemcomitans* serotypes in subgingival plaque from United States subjects. *Mol Oral Microbiol* (2010) 25(3):207–14. doi: 10.1111/j.2041-1014.2010.00567.x
12. van der Reijden WA, Bosch-Tijhof CJ, van der Velden U, van Winkelhoff AJ. Java project on periodontal diseases: serotype distribution of *Aggregatibacter actinomycetemcomitans* and serotype dynamics over an 8-year period. *J Clin Periodontol* (2008) 35(6):487–92. doi: 10.1111/j.1600-051X.2008.01218.x
13. Aberg CH, Sjodin B, Lakio L, Pussinen PJ, Johansson A, Claesson R. Presence of *Aggregatibacter actinomycetemcomitans* in young individuals: a 16-year clinical and microbiological follow-up study. *J Clin Periodontol* (2009) 36(10):815–22. doi: 10.1111/j.1600-051X.2009.01457.x
14. Kim TS, Frank P, Eickholz P, Eick S, Kim CK. Serotypes of *Aggregatibacter actinomycetemcomitans* in patients with different ethnic backgrounds. *J Periodontol* (2009) 80(12):2020–7. doi: 10.1902/jop.2009.090241
15. Cortelli JR, Aquino DR, Cortelli SC, Roman-Torres CV, Franco GC, Gomez RS, et al. *Aggregatibacter actinomycetemcomitans* serotypes infections and periodontal conditions: a two-way assessment. *Eur J Clin Microbiol Infect Dis* (2012) 31(7):1311–8. doi: 10.1007/s10096-011-1444-2
16. Zambon JJ, Slots J, Genco RJ. Serology of oral *Actinobacillus actinomycetemcomitans* and serotype distribution in human periodontal disease. *Infect Immun* (1983) 41(1):19–27. doi: 10.1128/IAI.41.1.19-27.1983
17. Yang HW, Asikainen S, Dogan B, Suda R, Lai CH. Relationship of *Actinobacillus actinomycetemcomitans* serotype *b to* aggressive periodontitis: frequency in pure cultured isolates. *J Periodontol* (2004) 75(4):592–9. doi: 10.1902/jop.2004.75.4.592
18. Monasterio G, Castillo F, Ibarra JP, Guevara J, Rojas L, Alvarez C, et al. Alveolar bone resorption and Th1/Th17-associated immune response

triggered during *Aggregatibacter actinomycetemcomitans*-induced experimental periodontitis are serotype-dependent. *J Periodontol* (2018) 89 (10):1249–61. doi: 10.1002/JPER.17-0563

19. Díaz-Zúñiga J, Melgar-Rodríguez S, Alvarez C, Monasterio G, Benitez A, Ciuchi P, et al. T-lymphocyte phenotype and function triggered by *Aggregatibacter actinomycetemcomitans* is serotype-dependent. *J Periodontal Res* (2015) 50(6):824–35. doi: 10.1111/jre.12270
20. Díaz-Zúñiga J, Melgar-Rodríguez S, Monasterio G, Pujol M, Rojas L, Alvarez C, et al. Differential human Th22-lymphocyte response triggered by *Aggregatibacter actinomycetemcomitans* serotypes. *Arch Oral Biol* (2017) 78:26–33. doi: 10.1016/j.archoralbio.2017.02.008
21. Díaz-Zúñiga J, Yáñez JP, Alvarez C, Melgar-Rodríguez S, Hernández M, Sanz M, et al. Serotype-dependent response of human dendritic cells stimulated with *Aggregatibacter actinomycetemcomitans*. *J Clin Periodontol* (2014) 41 (3):242–51. doi: 10.1111/jcpe.12205
22. Aberg CH, Haubek D, Kwamin F, Johansson A, Claesson R. Leukotoxic activity of *Aggregatibacter actinomycetemcomitans* and periodontal attachment loss. *PloS One* (2014) 9(8):e104095. doi: 10.1371/journal.pone.0104095
23. Lally ET, Kieba IR, Sato A, Green CL, Rosenbloom J, Korostoff J, et al. RTX toxins recognize a beta2 integrin on the surface of human target cells. *J Biol Chem* (1997) 272(48):30463–9. doi: 10.1074/jbc.272.48.30463
24. Perry MB, MacLean LL, Gmur R, Wilson ME. Characterization of the O-polysaccharide structure of lipopolysaccharide from *Actinobacillus actinomycetemcomitans* serotype *b*. *Infect Immun* (1996) 64(4):1215–9. doi: 10.1128/IAI.64.4.1215-1219.1996
25. Yoshida Y, Nakano Y, Yamashita Y, Koga T. Identification of a genetic locus essential for serotype *b*-specific antigen synthesis in *Actinobacillus actinomycetemcomitans*. *Infect Immun* (1998) 66(1):107–14. doi: 10.1128/IAI.66.1.107-114.1998
26. Tang G, Mintz KP. Glycosylation of the collagen adhesin EmaA of *Aggregatibacter actinomycetemcomitans is* dependent upon the lipopolysaccharide biosynthetic pathway. *J Bacteriol* (2010) 192(5):1395–404. doi: 10.1128/JB.01453-09
27. Tang G, Kawai T, Komatsuzawa H, Mintz KP. Lipopolysaccharides mediate leukotoxin secretion in *Aggregatibacter actinomycetemcomitans*. *Mol Oral Microbiol* (2012) 27(2):70–82. doi: 10.1111/j.2041-1014.2011.00632.x
28. Vernal R, Leon R, Herrera D, Garcia-Sanz JA, Sanz M. Variability in the response of human dendritic cells stimulated with *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*. *J Periodontal Res* (2008) 43 (6):689–97. doi: 10.1111/j.1600-0765.2007.01073.x
29. Díaz-Zúñiga J, Monasterio G, Alvarez C, Melgar-Rodríguez S, Benitez A, Ciuchi P, et al. Variability of the dendritic cell response triggered by different serotypes of *Aggregatibacter actinomycetemcomitans* or *Porphyromonas gingivalis* is toll-like receptor 2 (TLR2) or TLR4 dependent. *J Periodontol* (2015) 86(1):108–19. doi: 10.1902/jop.2014.140326
30. Marolda CL, Lahiry P, Vines E, Saldias S, Valvano MA. Micromethods for the characterization of lipid A-core and O-antigen lipopolysaccharide. *Methods Mol Biol* (2006) 347:237–52. doi: 10.1385/1-59745-167-3:237
31. Tsai CM, Frasch CE. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* (1982) 119(1):115–9. doi: 10.1016/0003-2697(82)90673-x
32. Monasterio G, Budini V, Fernandez B, Castillo F, Rojas C, Alvarez C, et al. IL-22-expressing $CD4^+$ AhR^+ T lymphocytes are associated with RANKL-mediated alveolar bone resorption during experimental periodontitis. *J Periodontal Res* (2019) 54(5):513–24. doi: 10.1111/jre.12654
33. Amano K, Nishihara T, Shibuya N, Noguchi T, Koga T. Immunochemical and structural characterization of a serotype-specific polysaccharide antigen from *Actinobacillus actinomycetemcomitans* Y4 (serotype *b*). *Infect Immun* (1989) 57(10):2942–6. doi: 10.1128/IAI.57.10.2942-2946.1989
34. Page RC, Sims TJ, Engel LD, Moncla BJ, Bainbridge B, Stray J, et al. The immunodominant outer membrane antigen of *Actinobacillus actinomycetemcomitans* is located in the serotype-specific high-molecular-mass carbohydrate moiety of lipopolysaccharide. *Infect Immun* (1991) 59(10):3451–62. doi: 10.1128/IAI.59.10.3451-3462.1991
35. Gaudino SJ, Kumar P. Cross-Talk between antigen presenting cells and T cells impacts intestinal homeostasis, bacterial infections, and tumorigenesis. *Front Immunol* (2019) 10:360. doi: 10.3389/fimmu.2019.00360
36. Janeway CAJr., Bottomly K. Signals and signs for lymphocyte responses. *Cell* (1994) 76(2):275–85. doi: 10.1016/0092-8674(94)90335-2
37. Hensel JA, Khattar V, Ashton R, Ponnazhagan S. Characterization of immune cell subtypes in three commonly used mouse strains reveals gender and strain-specific variations. *Lab Invest* (2019) 99(1):93–106. doi: 10.1038/s41374-018-0137-1
38. Garlet GP, Avila-Campos MJ, Milanezi CM, Ferreira BR, Silva JS. *Actinobacillus actinomycetemcomitans*-induced periodontal disease in mice: patterns of cytokine, chemokine, and chemokine receptor expression and leukocyte migration. *Microbes Infect* (2005) 7(4):738–47. doi: 10.1016/j.micinf.2005.01.012
39. Lima HR, Gelani V, Fernandes AP, Gasparoto TH, Torres SA, Santos CF, et al. The essential role of toll like receptor-4 in the control of *Aggregatibacter actinomycetemcomitans* infection in mice. *J Clin Periodontol* (2010) 37 (3):248–54. doi: 10.1111/j.1600-051X.2009.01531.x
40. Rogers JE, Li F, Coatney DD, Rossa C, Bronson P, Krieder JM, et al. *Actinobacillus actinomycetemcomitans* lipopolysaccharide-mediated experimental bone loss model for aggressive periodontitis. *J Periodontol* (2007) 78(3):550–8. doi: 10.1902/jop.2007.060321
41. Madeira MF, Queiroz-Junior CM, Cisalpino D, Werneck SM, Kikuchi H, Fujise O, et al. MyD88 is essential for alveolar bone loss induced by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide in mice. *Mol Oral Microbiol* (2013) 28(6):415–24. doi: 10.1111/omi.12034
42. Nakamura H, Fukusaki Y, Yoshimura A, Shiraishi C, Kishimoto M, Kaneko T, et al. Lack of Toll-like receptor 4 decreases lipopolysaccharide-induced bone resorption in C3H/HeJ mice *in vivo*. *Oral Microbiol Immunol* (2008) 23 (3):190–5. doi: 10.1111/j.1399-302X.2007.00410.x
43. Yamaguchi N, Kawasaki M, Yamashita Y, Nakashima K, Koga T. Role of the capsular polysaccharide-like serotype-specific antigen in resistance of *Actinobacillus actinomycetemcomitans* to phagocytosis by human polymorphonuclear leukocytes. *Infect Immun* (1995) 63(12):4589–94. doi: 10.1128/IAI.63.12.4589-4594.1995
44. Nishihara T, Ueda N, Amano K, Ishihara Y, Hayakawa H, Kuroyanagi T, et al. *Actinobacillus actinomycetemcomitans* Y4 capsular-polysaccharide-like polysaccharide promotes osteoclast-like cell formation by interleukin-1 alpha production in mouse marrow cultures. *Infect Immun* (1995) 63 (5):1893–8. doi: 10.1128/IAI.63.5.1893-1898.1995
45. Takahashi T, Nishihara T, Ishihara Y, Amano K, Shibuya N, Moro I, et al. Murine macrophage interleukin-1 release by capsularlike serotype-specific polysaccharide antigens of *Actinobacillus actinomycetemcomitans*. *Infect Immun* (1991) 59(1):18–23. doi: 10.1128/IAI.59.1.18-23.1991
46. Yamaguchi N, Yamashita Y, Ikeda D, Koga T. *Actinobacillus actinomycetemcomitans* serotype *b*-specific polysaccharide antigen stimulates production of chemotactic factors and inflammatory cytokines by human monocytes. *Infect Immun* (1996) 64(7):2563–70. doi: 10.1128/IAI.64.7.2563-2570.1996
47. Melgar-Rodriguez S, Diaz-Zuñiga J, Alvarez C, Rojas L, Monasterio G, Carvajal P, et al. Serotype *b* of *Aggregatibacter actinomycetemcomitans* increases osteoclast and memory T-lymphocyte activation. *Mol Oral Microbiol* (2016) 31(2):162–74. doi: 10.1111/omi.12112
48. Graves DT, Delima AJ, Assuma R, Amar S, Oates T, Cochran D. Interleukin-1 and tumor necrosis factor antagonists inhibit the progression of inflammatory cell infiltration toward alveolar bone in experimental periodontitis. *J Periodontol* (1998) 69(12):1419–25. doi: 10.1902/jop.1998.69.12.1419
49. Assuma R, Oates T, Cochran D, Amar S, Graves DT. IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. *J Immunol* (1998) 160(1):403–9.
50. Dutzan N, Kajikawa T, Abusleme L, Greenwell-Wild T, Zuazo CE, Ikeuchi T, et al. A dysbiotic microbiome triggers Th17 cells to mediate oral mucosal immunopathology in mice and humans. *Sci Transl Med* (2018) 10(463): eaat0797. doi: 10.1126/scitranslmed.aat0797
51. Gaffen SL, Moutsopoulos NM. Regulation of host-microbe interactions at oral mucosal barriers by type 17 immunity. *Sci Immunol* (2020) 5(43):eaau4594. doi: 10.1126/sciimmunol.aau4594
52. Garlet GP, Cardoso CR, Campanelli AP, Garlet TP, Avila-Campos MJ, Cunha FQ, et al. The essential role of IFN-gamma in the control of lethal *Aggregatibacter actinomycetemcomitans* infection in mice. *Microbes Infect* (2008) 10(5):489–96. doi: 10.1016/j.micinf.2008.01.010

53. Repeke CE, Ferreira SBJr., Claudino M, Silveira EM, de Assis GF, Avila-Campos MJ, et al. Evidences of the cooperative role of the chemokines CCL3, CCL4 and CCL5 and its receptors $CCR1^+$ and $CCR5^+$ in $RANKL^+$ cell migration throughout experimental periodontitis in mice. *Bone* (2010) 46 (4):1122–30. doi: 10.1016/j.bone.2009.12.030
54. Miyazaki Y, Nakayamada S, Kubo S, Nakano K, Iwata S, Miyagawa I, et al. Th22 cells promote osteoclast differentiation *via* production of IL-22 in rheumatoid arthritis. *Front Immunol* (2018) 9:2901. doi: 10.3389/fimmu.2018.02901
55. Zhao C, Chen Q, Yu S, Xu C, Li X, Zhang C, et al. Effect of interleukin-22 on osteogenic differentiation and the osteoclastogenic response of human periodontal ligament fibroblasts *in vitro*. *J Periodontol* (2020) 91(8):1085–97. doi: 10.1002/JPER.19-0470
56. Yang BH, Hagemann S, Mamareli P, Lauer U, Hoffmann U, Beckstette M, et al. $Foxp3^+$ T cells expressing RORγt represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. *Mucosal Immunol* (2016) 9(2):444–57. doi: 10.1038/mi.2015.74
57. Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help *via* APC activation. *J Exp Med* (1996) 184(2):747–52. doi: 10.1084/jem.184.2.747
58. Zughaier S, Agrawal S, Stephens DS, Pulendran B. Hexa-acylation and KDO (2)-glycosylation determine the specific immunostimulatory activity of *Neisseria meningitidis* lipid A for human monocyte derived dendritic cells. *Vaccine* (2006) 24(9):1291–7. doi: 10.1016/j.vaccine.2005.09.039
59. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive $Foxp3^+CD4^+$ regulatory T cells. *Proc Natl Acad Sci USA* (2008) 105(27):9331–6. doi: 10.1073/pnas.0710441105
60. van Panhuys N, Klauschen F. Germain RN. T-cell-receptor-dependent signal intensity dominantly controls $CD4^+$ T cell polarization *In Vivo*. *Immunity* (2014) 41(1):63–74. doi: 10.1016/j.immuni.2014.06.003
61. Darveau RP, Pham TT, Lemley K, Reife RA, Bainbridge BW, Coats SR, et al. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect Immun* (2004) 72(9):5041–51. doi: 10.1128/IAI.72.9.5041-5051.2004
62. Hirschfeld M, Weis JJ, Toshchakov V, Salkowski CA, Cody MJ, Ward DC, et al. Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. *Infect Immun* (2001) 69(3):1477–82. doi: 10.1128/IAI.69.3.1477-1482.2001
63. Kirikae T, Nitta T, Kirikae F, Suda Y, Kusumoto S, Qureshi N, et al. Lipopolysaccharides (LPS) of oral black-pigmented bacteria induce tumor necrosis factor production by LPS-refractory C3H/HeJ macrophages in a way different from that of *Salmonella* LPS. *Infect Immun* (1999) 67(4):1736–42. doi: 10.1128/IAI.67.4.1736-1742.1999
64. Tanamoto K, Azumi S, Haishima Y, Kumada H, Umemoto T. The lipid A moiety of *Porphyromonas gingivalis* lipopolysaccharide specifically mediates the activation of C3H/HeJ mice. *J Immunol* (1997) 158(9):4430–6.
65. Masoud H, Weintraub ST, Wang R, Cotter R, Holt SC. Investigation of the structure of lipid A from *Actinobacillus actinomycetemcomitans* strain Y4 and human clinical isolate PO 1021-7. *Eur J Biochem* (1991) 200(3):775–81. doi: 10.1111/j.1432-1033.1991.tb16244.x
66. Brondz I, Olsen I. Chemical differences in lipopolysaccharides from *Actinobacillus (Haemophilus) actinomycetemcomitans* and *Haemophilus aphrophilus*: clues to differences in periodontopathogenic potential and taxonomic distinction. *Infect Immun* (1989) 57(10):3106–9. doi: 10.1128/IAI.57.10.3106-3109.1989
67. Vega BA, Belinka BA Jr., Kachlany SC. *Aggregatibacter actinomycetemcomitans* leukotoxin (LtxA; Leukothera®): mechanisms of action and therapeutic applications. *Toxins (Basel)* (2019) 11(9):489. doi: 10.3390/toxins11090489
68. Konig MF, Abusleme L, Reinholdt J, Palmer RJ, Teles RP, Sampson K, et al. *Aggregatibacter actinomycetemcomitans*-induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis. *Sci Transl Med* (2016) 8(369):369ra176. doi: 10.1126/scitranslmed.aaj1921
69. Jia R, Kurita-Ochiai T, Oguchi S, Yamamoto M. Periodontal pathogen accelerates lipid peroxidation and atherosclerosis. *J Dent Res* (2013) 92 (3):247–52. doi: 10.1177/0022034513475625
70. Eke PI, Dye BA, Wei L, Slade GD, Thornton-Evans GO, Borgnakke WS, et al. Update on prevalence of periodontitis in adults in the United States: NHANES 2009 to 2012. *J Periodontol* (2015) 86(5):611–22. doi: 10.1902/jop.2015.140520
71. Shiau HJ, Reynolds MA. Sex differences in destructive periodontal disease: exploring the biologic basis. *J Periodontol* (2010) 81(11):1505–17. doi: 10.1902/jop.2010.100045
72. Shiau HJ, Aichelmann-Reidy ME, Reynolds MA. Influence of sex steroids on inflammation and bone metabolism. *Periodontol 2000* (2014) 64(1):81–94. doi: 10.1111/prd.12033
73. Duan X, Gleason RC, Li F, Hosur KB, Duan X, Huang D, et al. Sex dimorphism in periodontitis in animal models. *J Periodontal Res* (2016) 51 (2):196–202. doi: 10.1111/jre.12298

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Potential Role of Phosphoglycerol Dihydroceramide Produced by Periodontal Pathogen *Porphyromonas gingivalis* in the Pathogenesis of Alzheimer's Disease

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Background: Among different types of sphingolipids produced by human cells, the possible engagement of ceramide species in the pathogenesis of Alzheimer's disease (AD) has attracted recent attention. While ceramides are primarily generated by *de novo* synthesis in mammalian cells, only a limited number of bacterial species, produce ceramides, including phosphoglycerol dihydroceramide (PGDHC) that is produced by the key periodontal pathogen *Porphyromonas gingivalis*. Emerging evidence indicates that virulence factors produced by *P. gingivalis*, such as lipopolysaccharide and gingipain, may be engaged in the initiation and/or progression of AD. However, the potential role of PGDHC in the pathogenesis of AD remains unknown. Therefore, the aim of this study was to evaluate the influence of PGDHC on hallmark findings in AD.

Material and Methods: CHO-7WD10 and SH-SY-5Y cells were exposed to PGDHC and lipopolysaccharide (LPS) isolated from *P. gingivalis*. Soluble Aβ42 peptide, amyloid precursor protein (APP), phosphorylated tau and senescence-associated secretory phenotype (SASP) factors were quantified using ELISA and Western blot assays.

Results: Our results indicate that *P. gingivalis (Pg)*-derived PGDHC, but not *Pg*-LPS, upregulated secretion of soluble Aβ42 peptide and expression of APP in CHO-7WD10 cells. Furthermore, hyperphosphorylation of tau protein was observed in SH-SY-5Y cells in response to PGDHC lipid. In contrast, *Pg*-LPS had little, or no significant effect on the tau phosphorylation induced in SH-SY-5Y cells. However, both PGDHC and *Pg*-LPS contributed to the senescence of SH-SY5Y cells as indicated by the production of senescence-associated secretory phenotype (SASP) markers, including beta-galactosidase, cathepsin B (CtsB), and pro-inflammatory cytokines TNF-α, and IL-6. Additionally, PGDHC diminished expression of the senescence-protection marker sirtuin-1 in SH-SY-5Y cells.

Conclusions: Altogether, our results indicate that *P. gingivalis*-derived PGDHC ceramide promotes amyloidogenesis and hyperphosphorylation, as well as the production of SASP factors. Thus, PGDHC may represent a novel class of bacterial-derived virulence factors for AD associated with periodontitis.

Keywords: Alzheimer's Disease, *Porphyromonas gingivalis*, dihydroceramides, amyloid precursor protein, tau protein, cellular senescence, senescence-associated secretory phenotype

INTRODUCTION

Alzheimer's disease (AD) is a multifactorial, highly heterogeneous, and complex neurodegenerative disorder that affects memory and cognitive functions leading to total dependence on nursing care at an advanced stage. Approximately 35.6 million patients are affected by AD worldwide and about 4.6 million new cases are added each year, causing enormous societal and economic burden (1, 2). It is commonly accepted that elevated amounts of aggregated Aβ peptides and hyperphosphorylated tau protein lead to deposition of extracellular amyloid plaques and intracellular neurofibrillary tangles in the brain of AD patients, making them hallmark features of AD neuropathology (3). The growing evidence suggests that age is the most prevalent risk factor for AD (4, 5). Although the age-associated gut bacterial dysbiosis is significantly correlated with the pathogenesis of AD (6), there is limited knowledge about the impact of oral bacteria on aging-associated AD.

The oral Gram-negative anaerobe, *Porphyromonas gingivalis*, is considered to be a keystone pathogen in chronic periodontitis (7–10). It is also well-documented that *P. gingivalis* is a contributory factor for various systemic diseases associated with aging, including type-II diabetes, and cardiovascular diseases (11). Furthermore, presence of *P. gingivalis* in AD brains (12, 13), as well as detection of elevated levels of IgG against *P. gingivalis* in periodontitis patients with AD, implicates a potential contributory role of this periodontal bacteria in the pathogenesis of AD (14).

P. gingivalis produces a wide variety of virulence factors of lipid origin, including lipopolysaccharide (LPS) and novel sphingolipids termed phosphoglycerol dihydroceramide (PGDHC) and phosphoethanolamine dihydroceramide (PEDHC) (15). Although ligation of *Pg*-LPS and PEDHC with Toll-Like Receptor (TLR) 2 and TLR4 elicits a strong inflammatory signaling induced in young mice, various published studies indicated that TLR function may be impaired in the context of aging (16–18). Furthermore, it was also recently demonstrated that *P.g*-LPS had little, or no, effect on the promotion of periodontitis inflammation induced in aged mice (19). We, however, reported that PGDHC ceramide promotes inflammation in a manner independent of TLRs (20), indicating that PGDHC may also represent a novel virulence risk factor that contributes to various age-related disorders, including periodontitis and AD.

Emerging evidence has indicated that among different sphingolipids, the levels of mammalian ceramide species were significantly elevated in brains of patients with more than one neuropathologic abnormality compared to the age-matched neurologically normal group of people (21). Although bacterial dihydroceramides, including those derived by *P. gingivalis*, share basic structural characteristics with mammalian ceramides, sphingolipid production by bacteria was thought to be a rare occurrence because only a limited number of gut and oral bacterial species can synthesize ceramides *de novo* (22–24). Nonetheless, because no studies have yet examined the role of ceramides produced by oral bacterial in the pathogenesis of AD, it remains unclear whether *P. gingivalis*-derived PGDHC contributes to the onset or progression of AD. Therefore, this study aimed to evaluate the potential involvement of PGDHC in the amyloidogenic processing of amyloid precursor protein (APP), hyperphosphorylation of tau, and cellular senescence, as key features of AD pathogenesis.

MATERIAL AND METHODS

Cell Cultures

Chinese hamster ovary-7WD10 (CHO-7WD10) cells stably expressing human wild-type amyloid precursor protein 751 (APP751WT) were cultured in DMEM media (Corning) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2mM L-glutamine. SH-SY5Y human neuroblastoma cells were cultured in 1:1 mixture of DMEM: F12 media with the same supplements as that of CHO-7WD10 cells.

PGDHC and LPS From *Porphyromonas gingivalis*

PGDHC was isolated from *Porphyromonas gingivalis* (ATTC strain #33277) as previously described (20). For biological experiments, PGDHC was sonicated (2 min, 3 W) in phosphate-buffered saline (PBS) to achieve a concentration of 100 μg/ml. Ultrapure *Pg*-LPS was purchased from InvivoGen and prepared according to the manufacturer's recommendation.

Cytotoxicity Assays

CHO-7WD10 and SH-SY-5Y cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and exposed to several concentrations of *Pg*-LPS and PGDHC (0, 1, 5, 8, 10 μg/ml). After 24 h of incubation, WST-1 metabolic activity assay (Sigma Aldrich) was employed according to the manufacturer's instructions.

Quantification of Soluble Aβ42

CHO-7WD10 cells were seeded in a 6-well plate at a density of 1×10^6 cells/well and cultured in the presence or absence of *Pg*-LPS or PGDHC for 48 h. Culture supernatants were collected and the amount of soluble Aβ42 was quantified using a commercial sandwich ELISA kit from Thermofisher.

Quantification of β-Galactosidase Activity in SH-SY-5Y Cells

β-galactosidase activity was evaluated using a commercial senescence β-galactosidase staining kit (Cell Signalling Technology) according to the manufacturer's recommendation. Staining-patterns of cells in the culture well were acquired by a 20x objective lens using an EVOS cell imaging system under bright-field illumination.

Western Blot Analysis

CHO-7WD10 and SH-SY-5Y cells were seeded at 1×10^6 cells/well in a six-well plates and stimulated with various concentrations of PGDHC and Pg-LPS as listed for the cytotoxicity assay. After 48 h of stimulation, cells were lysed in the lysis buffer (Thermofisher) and protein concentration was measured using the BCA kit (Pierce). Next, proteins were separated using SDS/PAGE (Bolt 12% gel) electrophoresis, transferred onto a nitrocellulose (NC) membrane and blocked using iBlot2 (Thermofisher). The anti-mouse CT15 polyclonal antibody (1:500) (Calbiochem) was used for detection of full-length APP in CHO-7WD10.

To detect phosphorylated-Tau (p-Tau), sirtuin-1, and cathepsin B in SH-SY5Y cells, rabbit anti-p-Tau (Ser^{396}), and -mouse AT1000 (Thr^{212}/Ser^{214}), - Sirt-1 and -cathepsin B polyclonal antibodies (1:1,000; Thermofisher) were used, respectively. The anti-human β-actin antibody (cat # 1:2,000; CST) was used to detect the levels of β-actin as a loading control. Finally, the membranes were washed with tris-buffered saline (TBS) containing 0.05% Tween 20 and then processed using horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies (Amersham Pharmacia Biotech) followed by enhanced chemiluminescence detection (ThermoFisher). The signal intensity of Western blots was quantified using Image J.

Real-Time PCR Analysis of Gene Expression

RNA was isolated using the PureLink™ RNA Mini Kit (Ambion, Life Technologies, USA) according to manufacturer's instructions. Altogether, 1 μg of RNA was reverse transcribed with the Verso cDNA Synthesis Kit (Thermo Scientific). Gene expression was quantified using PowerUp™ Sybr™ Green Master Mix (Applied Biosystems Diagnostics) in the AriaMx Real-time PCR System (Agilent). Data were analyzed by the ΔΔCt method normalized to β-actin as the internal reference gene. Primer sequences are available upon request.

Statistical Analysis

Significant differences in quantitative data were determined by one-way analysis of variance (ANOVA) followed by Tukey's posthoc test using the paleontological statistics software (PAST) version 4.02 and p values ≤ 0.05 were considered significant. The data are displayed as means ± standard deviation (SD).

RESULTS

PGDHC Enhances Secretion of Amyloid-Beta (Aβ) in CHO-7WD10 Cells

Since genetic, biochemical, and pathological evidence has strongly implicated that Aβ plays an early and crucial role in AD pathogenesis (25), we first tested whether PGDHC in comparison to *Pg*-LPS, exacerbate amyloidogenic processing of APP, using CHO cells stably expressing human APP751WT protein (CHO-7WD10) *in vitro*. When the concentrations of both *Pg*-LPS and PGDHC were greater than 5 μg/ml, our results showed that the viability of CHO-7WD10 cells was significantly reduced. Thus, CHO-7WD10 cells exposed to 8 and 10 μg/ml of PGDHC and *Pg*-LPS were excluded from the further examinations.

According to our results, exposure of CHO-7WD10 cells to PGDHC, but not to *Pg*-LPS, significantly elevated the release of Aβ42 peptide in a dose-dependent manner (**Figures 1A, B**). Next, to detect the level of APP using a Western blot assay, lysates were prepared from the same cells that were used for Aβ42 quantitation after treatment with different concentrations of *Pg*-LPS and PGDHC. *Pg*-LPS had no or little effects on the APP levels in CHO-7WD10 cells (**Figures 2A, B**). By contrast, PGDHC significantly elevated the levels of APP in CHO-7WD10 cells when compared to the control, non-treated cells (**Figures 2C, D**). These observations suggested that PGDHC, but not *Pg*-LPS, enhances secretion of Aβ from CHO-7WD10 cells *in vitro*.

PGDHC Induces the Site-Specific Phosphorylation of Tau (p-Tau) in SH-SY-5Y Cells

Because published evidence demonstrated that the hyperphosphorylation of Tau protein was significantly upregulated in the hippocampi of AD patients (26), we next wanted to assess the p-Tau status in SH-SY-5Y cells exposed to various concentrations of *Pg*-LPS and PGDHC using two antibodies that recognize hyperphosphorylated tau at Ser^{396} and Thr^{212}/Ser^{214} sites by Western blot assay. We observed that p-Tau at Ser^{396} was significantly increased after treatment with PGDHC when compared to control cells (**Figures 3A, B**). Furthermore, PGDHC also significantly upregulated p-Tau at Thr^{212}/Ser^{214} in a dose-dependent manner compared to the control cells (**Figures 3C, D**). However, *Pg*-LPS did not induce p-Tau at either Ser^{396} or Thr^{212}/Ser^{214} loci in SH-SY-5Y cells (**Figures 3A–D**). These results indicate that PGDHC may

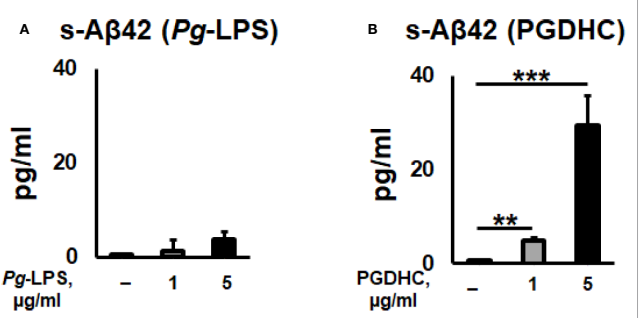

FIGURE 1 | *Porphyromonas gingivalis*-derived phosphoglycerol dihydroceramide (PGDHC) promotes Aβ-42 secretion from Chinese hamster ovary (CHO) cells stably expressing human wild-type amyloid precursor protein 751 protein (CHO-7WD10). CHO-7WD10 cells were exposed to different concentrations of *Pg*-LPS **(A)** or PGDHC **(B)** for 48 h. Then, the conditioned media were collected and analyzed by ELISA. N = 4 samples/condition. **p < 0.01, ***p < 0.001.

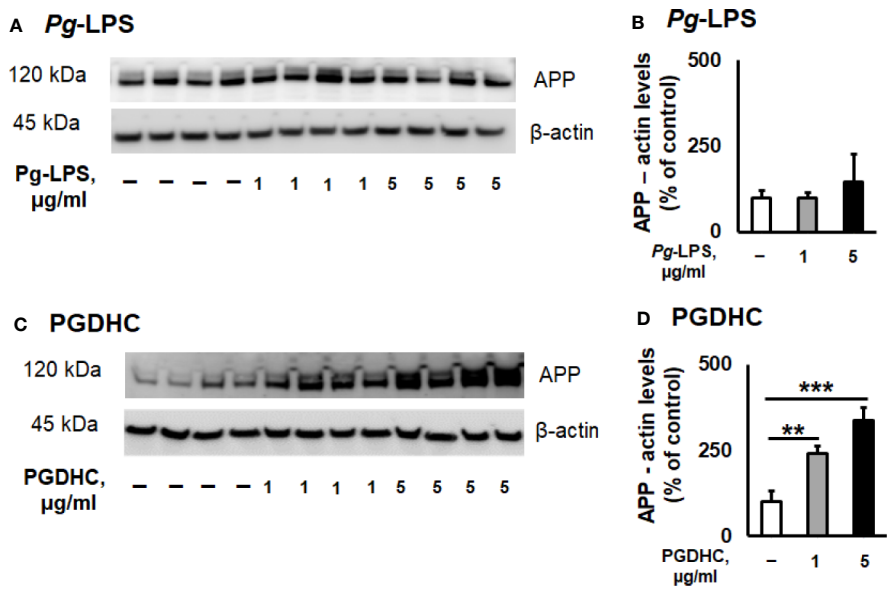

FIGURE 2 | Phosphoglycerol dihydroceramide (PGDHC) amplifies the level of amyloid precursor protein (APP) in lysates of CHO-7WD10 cells. CHO-7WD10 cells were stimulated with various concentrations of *Pg*-LPS **(A, B)** or PGDHC **(C, D)** for 48 h and lysates were then prepared and analyzed by Western blot. The signal quantification was carried out using Image J. ANOVA with Tukey's post-hoc test was used to evaluate the statistical significance. N = 4 samples/condition.
**p < 0.01, ***p < 0.001.

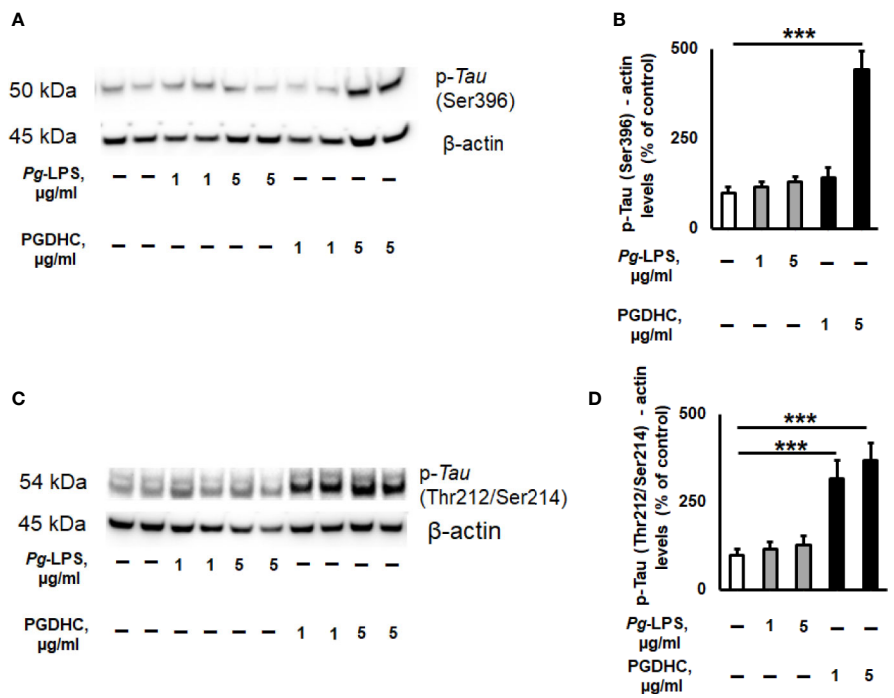

FIGURE 3 | The effects of phosphoglycerol dihydroceramide (PGDHC) and lipopolysaccharide (LPS) isolated from *Porphyromonas gingivalis* on hyperphosphorylation of tau protein in SH-SY-5Y cells *in vitro*. Representative images and quantification of tau phosphorylation at Ser396 **(A, B)** and Thr212/Ser214 **(C, D)** loci in SH-SY-5Y cells after exposure to *P. gingivalis*-LPS (Pg-LPS) and PGDHC for 48 h. Cell lysates were prepared and analyzed by Western blot. Western blot signal quantification was done using Image J. ANOVA with Tukey's post-hoc test was used to evaluate the statistical significance. n=4 samples/condition.
***p < 0.001.

play an important role in the hyperphosphorylation of Tau protein, in addition to enhancing the secretion of Aβ.

PGDHC Promotes the Development of Senescence-Associated Secretory Phenotype in SH-SY-5Y Cells

Since the cellular senescence of neurons is tightly connected with AD pathogenesis as well as other neurodegenerative diseases (27, 28), we next examined whether PGDHC or *Pg*-LPS elevated expression of some senescence-associated secretory phenotype (SASP) factors, including β-galactosidase, cathepsin B (CtsB) cysteine, and TNF-α and IL-6 pro-inflammatory cytokines in SH-SY5Y cells *in vitro*. We observed that exposure of SH-SY-5Y cells to *Pg*-LPS and PGDHC, both significantly elevated activity of senescence-associated β-galactosidase (**Figures 4A, B**) and CtsB (**Figures 4B, C**) compared with control cells. Further, expression patterns of pro-inflammatory TNF-α and IL-6 mRNAs were also significantly elevated in response to *Pg*-LPS and PGDHC (**Figures 4D, E**). On the other hand, expression of a senescence protection marker, sirtuin-1 (Sirt-1) was significantly diminished in SH-SY-5Y cells in response to *P. gingivalis*-derived PGDHC and *Pg*-LPS (**Figures 5A, B**). Therefore, these results indicate that persistent exposure of neurons to either PGDHC or *Pg*-LPS may induce phenotypes reminiscent of cellular senescence.

DISCUSSION

In this study, we aimed to examine the potential impact of phosphoglycerol dihydroceramide (PGDHC) isolated from the periodontal pathogen *Porphyromonas gingivalis* on the key features of AD pathogenesis, including amyloidogenesis, phosphorylation of tau protein, and cellular senescence, using *in vitro* models of AD. To the best of our knowledge, this is the first study reporting that PGDHC

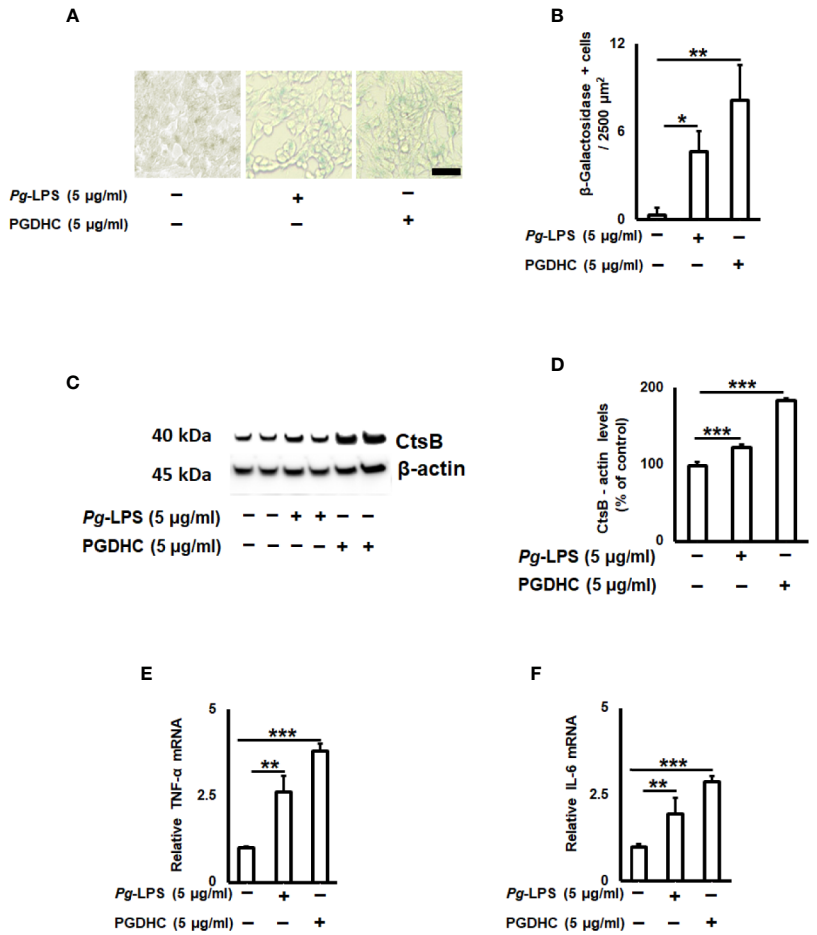

FIGURE 4 | Quantification of senescence-associated secretory phenotype (SASP) factors on P. gingivalis-LPS (*Pg*-LPS) and phosphoglycerol dihydroceramide (PGDHC)-stimulated SH-SY-5Y cells *in vitro*. Representative images **(A)** and quantification **(B)** of β-galactosidase activity. The number of blue β-galactosidase positive senescent cells was quantified microscopically. Scale bar is 50 μm. Representative signals **(C)** and quantification **(D)** of cathepsin B. Expression patterns of TNF-a **(E)** and IL-6 **(F)** mRNAs in SH-SY-5Y cells exposed to *Pg*-LPS and PGDHC. ANOVA with Tukey's post-hoc test was used to evaluate the statistical significance. N = 4 samples/condition. *p < 0.05, **p < 0.01, ***p < 0.001.

significantly upregulates amyloidogenesis in CHO-7WD10 cells. Our results also indicate that PGDHC elevates tau phosphorylation and expression of senescence-associated phenotype (SASP) factors in SH-SY-5Y neuronal cells *in vitro*.

Accumulating lines of evidence support the conclusion that ceramide sphingolipids are important structural and bioactive signaling molecules in mammalian cells, with significant roles in the regulation of cell apoptosis, senescence, and autophagy, leading to the development of AD pathogenesis as well as other age-related neurodegenerative disorders (29, 30). On the other hand, it was reported that a limited number of human gut and oral bacteria belonging to Bacteroidetes phylum are also able to produce dihydroceramides that upregulate intracellular host ceramide levels (23, 31). Further, bacterial-derived sphingolipids have been shown to signal *via* inflammation-related pathways in colon and gingival tissues (23, 24, 32). Important to this study, the gut Bacteroidetes species were detected at higher levels in AD patients compared to healthy controls (33). However, the role of dihydroceramides produced by oral *Bacteroides* spp. bacteria in the pathogenesis of AD has not been evaluated.

To date, unique dihydroceramides with non-mammalian structure, termed PGDHC and PEDHC, have been detected in three oral Gram-negative bacterial species associated with chronic periodontal disease, including *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Prevotella intermedia* (24). These periodontal pathogens also produce several virulence factors, including LPS, gingipain, and lipids, which promote tissue inflammation, loss of connective tissue attachment, and bone loss (34, 35). It is important to mention that a recently published observation suggested that *P. gingivalis*-derived dihydroceramides are critical to the long-term persistence and presentation of other virulence factors, such as gingipains and polysaccharides (36).

Among periodontal bacteria species, *P. gingivalis* and its virulence factors were identified as significant risk factors for developing AD hallmarks (15, 37, 38). Increasing genetic, biochemical, and pathological evidence strongly implies that both amyloidogenesis and tauopathy play a crucial pathological role in brains of AD patients (25). It is commonly accepted that amyloidogenesis is associated with the production of Aβ peptides from its precursor protein APP by the consecutive actions of β- and γ-secretases, while tauopathy shows hyper-phosphorylation of tau protein in the brain of AD patients (39). A previous study reported that enhanced levels of intracellular mammalian ceramides directly affect the accumulation of *A*β peptides and p-Tau *in vitro* as well as *in vivo* (30). Here, we demonstrated that *P. gingivalis*-derived PGDHC increased the expression of APP protein and production of soluble *A*β42 peptide in CHO7W10 cells (**Figure 1**) as well as hyperphosphorylation of tau protein in SH-5Y-SY cells *in vitro* (**Figure 2**), indicating a potential contribution of oral bacterial-derived dihydroceramides in amyloidogenesis and tauopathy. By contrast, we observed no or minimal effects of ultrapure *Pg*-LPS on the release of soluble *Aβ*, and tau phosphorylation. These data contradict with earlier reports indicating that *Pg*-LPS promoted accumulation of Aβ and p-Tau *in vitro* as well as in the brains of young and mid-age APP-transgenic mice and their wild type (37, 40–42).

Besides the role of Aβ and p-Tau in the AD pathogenesis, a relationship between cellular senescence and AD may represent an additional hallmark in the context of aging (27, 43). More specifically, several groups have highlighted the potential beneficial effects of eliminating senescent cells on the AD-associated neurodegeneration (44–46). It was also demonstrated that elevated activities of lysosomal β-galactosidase and neurodegenerative cathepsin B (CtsB) as well as secretion of various pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), collectively termed as senescence-associated secretory phenotype (SASP) factors, directly correlate with cellular senescence and aging (47). Findings from our study also confirmed that both PGDHC and *Pg*-LPS exacerbate the activity of senescence-associated β-galactosidase as well as the levels of CtsB protein in SH-SY-5Y cells *in vitro* (**Figure 4**). In addition, we also demonstrated that PGDHC and *Pg*-LPS upregulated the expression of pro-inflammatory TNF-α and IL-6, suggesting the possible impact of *P. gingivalis*-derived virulence factors on the promotion of neuronal senescence (**Figure 4**). These data agree with earlier published observations indicating that LPS isolated from *P. gingvalis* induces premature cellular senescence (48) as well as promotes

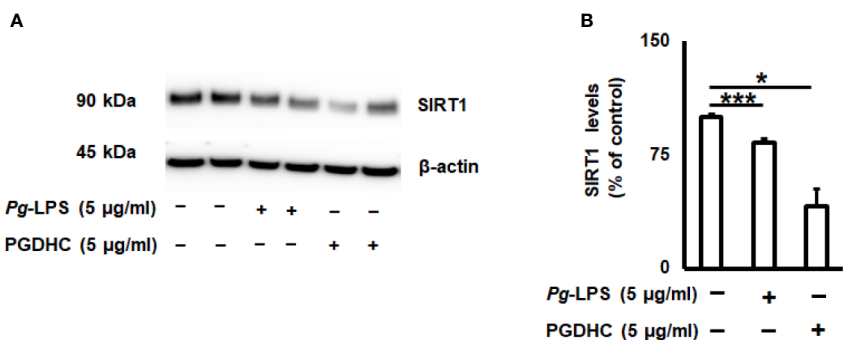

FIGURE 5 | *Porphyromonas gingivalis*-derived phosphoglycerol dihydroceramide (PGDHC) abrogates expression of the senesce-protection sirtuin-1 (Sirt-1) in SH-SY-5Y cells *in vitro*. SH-SY-5Y cells were exposed either to Pg-LPS or PGDHC for 48 h and then the levels of Sirt-1 were evaluated by Western blot **(A, B)**. ANOVA with Tukey's posthoc test was used to evaluate the statistical significance. N = 4 samples/condition. *p < 0.05, ***p < 0.001.

development of AD-like phenotypes in mice *via* a CtsB-dependent manner (42). Also, the pathological role of mammalian ceramides in the promotion of cellular senescence and aging has been well documented (49).

While earlier studies reported that elevated production of SASPs contributes to the AD pathology, it was suggested that the age-protection NAD^+-dependent sirtuin enzymes display beneficial effects in aging-related disorders, including AD (50). A positive correlation between Sirt1 activity and reduction of Aβ plaques and tauopathies was established in various animal models of AD (51–54). Here, we also confirmed that PGDHC as well as *Pg*-LPS both diminished the amount of Sirt-1 protein in SH-SY-5Y cells (**Figure 5**), indicating that *P. gingivalis* may downregulate the expression of aging protection markers in the human brain. Since elevated expression of Sirt-1 reduces cellular senescence, the potential effect of sirtuin agonists to abrogate the negative effects of PGDHC in the pathology of AD warrants further examination.

CONCLUSION

Collectively, the findings from this study indicate that PGDHC sphingolipid, isolated from the periodontal pathogen *P. gingivalis*, upregulated secretion of soluble Aβ42 peptide and expression of APP in CHO-7WD10 cells. Moreover, elevated hyperphosphorylation of tau protein (p-Tau) was observed in human neuronal SH-SY-5Y cells in response to PGDHC. Furthermore, we found that PGDHC contributed to the cellular senescence of SH-SY-5Y cells *via* 1) production of SASP markers, including beta-galactosidase, cathepsin B (CtsB), and pro-inflammatory cytokines TNF-α and IL-6, and 2) downregulation of the senescence-protection marker sirtuin-1 (Sirt-1). Altogether, these data indicate that PGDHC may be a novel class of bacterial-derived virulence factor for AD, finding which lay the groundwork for future studies, evaluating the molecular mechanisms of AD pathology associated with periodontitis.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

AM and ML contributed to the conception, design of the study, and wrote the manuscript. CY, AH, JA, and CR performed all the experiments. RD, TK, and FN 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.2020.591571/full#supplementary-material

REFERENCES

1. Wimo A, Winblad B, Jonsson L. The worldwide societal costs of dementia: Estimates for 2009. *Alzheimers Dement* (2010) 6(2):98–103. doi: 10.1016/j.jalz.2010.01.010
2. Wimo A, Guerchet M, Ali GC, Wu YT, Prina AM, Winblad B, et al. The worldwide costs of dementia 2015 and comparisons with 2010. *Alzheimers Dement* (2017) 13(1):1–7. doi: 10.1016/j.jalz.2016.07.150
3. Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* (2001) 81(2):741–66. doi: 10.1152/physrev.2001.81.2.741
4. Qiu C, Kivipelto M, von Strauss E. Epidemiology of Alzheimer's disease: occurrence, determinants, and strategies toward intervention. *Dialogues Clin Neurosci* (2009) 11(2):111–28.
5. Corrada MM, Brookmeyer R, Paganini-Hill A, Berlau D, Kawas CH. Dementia incidence continues to increase with age in the oldest old: the 90 + study. *Ann Neurol* (2010) 67(1):114–21. doi: 10.1002/ana.21915
6. Cerovic M, Forloni G, Balducci C. Neuroinflammation and the Gut Microbiota: Possible Alternative Therapeutic Targets to Counteract Alzheimer's Disease? *Front Aging Neurosci* (2019) 11:284. doi: 10.3389/fnagi.2019.00284
7. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, et al. Bacterial diversity in human subgingival plaque. *J Bacteriol* (2001) 183 (12):3770–83. doi: 10.1128/JB.183.12.3770-3783.2001
8. Ozmeric N, Preus NR, Olsen I. Genetic diversity of Porphyromonas gingivalis and its possible importance to pathogenicity. *Acta Odontol Scand* (2000) 58 (4):183–7. doi: 10.1080/000163500429190
9. Holt SC, Ebersole JL. Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol 2000* (2005) 38:72–122. doi: 10.1111/j.1600-0757.2005.00113.x
10. Wu Y, Dong G, Xiao W, Xiao E, Miao F, Syverson A, et al. Effect of Aging on Periodontal Inflammation, Microbial Colonization, and Disease Susceptibility. *J Dent Res* (2016) 95(4):460–6. doi: 10.1177/0022034515625962
11. Carter CJ, France J, Crean S, Singhrao SK. The Porphyromonas gingivalis/Host Interactome Shows Enrichment in GWASdb Genes Related to Alzheimer's Disease, Diabetes and Cardiovascular Diseases. *Front Aging Neurosci* (2017) 9:408. doi: 10.3389/fnagi.2017.00408
12. Riviere GR, Riviere KH, Smith KS. Molecular and immunological evidence of oral Treponema in the human brain and their association with Alzheimer's disease. *Oral Microbiol Immunol* (2002) 17(2):113–8. doi: 10.1046/j.0902-0055.2001.00100.x
13. Poole S, Singhrao SK, Kesavalu L, Curtis MA, Crean S. Determining the presence of periodontopathic virulence factors in short-term postmortem Alzheimer's disease brain tissue. *J Alzheimers Dis* (2013) 36(4):665–77. doi: 10.3233/JAD-121918
14. Kamer AR, Craig RG, Pirraglia E, Dasanayake AP, Norman RG, Boylan RJ, et al. TNF-alpha and antibodies to periodontal bacteria discriminate between

Alzheimer's disease patients and normal subjects. *J Neuroimmunol* (2009) 216(1-2):92–7. doi: 10.1016/j.jneuroim.2009.08.013
15. Singhrao SK, Harding A, Poole S, Kesavalu L, Crean S. Porphyromonas gingivalis Periodontal Infection and Its Putative Links with Alzheimer's Disease. *Mediators Inflammation* (2015) 2015:137357. doi: 10.1155/2015/137357
16. Shaik-Dasthagirisaheb YB, Huang N, Weinberg EO, Shen SS, Genco CA, Gibson FC 3rd. Aging and contribution of MyD88 and TRIF to expression of TLR pathway-associated genes following stimulation with Porphyromonas gingivalis. *J Periodontal Res* (2015) 50(1):89–102. doi: 10.1111/jre.12185
17. Shaw AC, Panda A, Joshi SR, Qian F, Allore HG, Montgomery RR. Dysregulation of human Toll-like receptor function in aging. *Ageing Res Rev* (2011) 10(3):346–53. doi: 10.1016/j.arr.2010.10.007
18. Dunston CR, Griffiths HR. The effect of ageing on macrophage Toll-like receptor-mediated responses in the fight against pathogens. *Clin Exp Immunol* (2010) 161(3):407–16. doi: 10.1111/j.1365-2249.2010.04213.x
19. Akkaoui J, Yamada C, Duarte C, Ho A, Vardar-Sengul S, Kawai T, et al. Contribution of Porphyromonas gingivalis lipopolysaccharide to experimental periodontitis in relation to aging. *Geroscience* (2020). doi: 10.1007/s11357-020-00258-1
20. Kanzaki H, Movila A, Kayal R, Napimoga MH, Egashira K, Dewhirst F, et al. Phosphoglycerol dihydroceramide, a distinctive ceramide produced by Porphyromonas gingivalis, promotes RANKL-induced osteoclastogenesis by acting on non-muscle myosin II-A (Myh9), an osteoclast cell fusion regulatory factor. *Biochim Biophys Acta Mol Cell Biol Lipids* (2017) 1862(5):452–62. doi: 10.1016/j.bbalip.2017.01.008
21. Filippov V, Song MA, Zhang K, Vinters HV, Tung S, Kirsch WM, et al. Increased ceramide in brains with Alzheimer's and other neurodegenerative diseases. *J Alzheimers Dis* (2012) 29(3):537–47. doi: 10.3233/JAD-2011-111202
22. Olsen I, Nichols FC. Are Sphingolipids and Serine Dipeptide Lipids Underestimated Virulence Factors of Porphyromonas gingivalis? *Infect Immun* (2018) 86(7):1–10. doi: 10.1128/IAI.00035-18
23. Johnson EL, Heaver SL, Waters JL, Kim BI, Bretin A, Goodman AL, et al. Sphingolipids produced by gut bacteria enter host metabolic pathways impacting ceramide levels. *Nat Commun* (2020) 11(1):2471. doi: 10.1038/s41467-020-16274-w
24. Nichols FC, Yao X, Bajrami B, Downes J, Finegold SM, Knee E, et al. Phosphorylated dihydroceramides from common human bacteria are recovered in human tissues. *PloS One* (2011) 6(2):e16771. doi: 10.1371/journal.pone.0016771
25. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* (2002) 297(5580):353–6. doi: 10.1126/science.1072994
26. Sze CI, Su M, Pugazhenthi S, Jambal P, Hsu LJ, Heath J, et al. Down-regulation of WW domain-containing oxidoreductase induces Tau phosphorylation in vitro. A potential role in Alzheimer's disease. *J Biol Chem* (2004) 279 (29):30498–506. doi: 10.1074/jbc.M401399200
27. Saez-Atienzar S, Masliah E. Cellular senescence and Alzheimer disease: the egg and the chicken scenario. *Nat Rev Neurosci* (2020) 21(8):433–44. doi: 10.1038/s41583-020-0325-z
28. Kritsilis M, Rizou SV, Koutsoudaki PN, Evangelou K, Gorgoulis VG, Papadopoulos D. Ageing, Cellular Senescence and Neurodegenerative Disease. *Int J Mol Sci* (2018) 19(10):1–37. doi: 10.3390/ijms19102937
29. Mielke MM, Bandaru VV, Haughey NJ, Xia J, Fried LP, Yasar S, et al. Serum ceramides increase the risk of Alzheimer disease: the Women's Health and Aging Study II. *Neurology* (2012) 79(7):633–41. doi: 10.1212/WNL.0b013e318264e380
30. Jazvinscak Jembrek M, Hof PR, Simic G. Ceramides in Alzheimer's Disease: Key Mediators of Neuronal Apoptosis Induced by Oxidative Stress and Abeta Accumulation. *Oxid Med Cell Longev* (2015) 2015:346783. doi: 10.1155/2015/346783
31. An D, Oh SF, Olszak T, Neves JF, Avci FY, Erturk-Hasdemir D, et al. Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. *Cell* (2014) 156(1-2):123–33. doi: 10.1016/j.cell.2013.11.042
32. Brown EM, Ke X, Hitchcock D, Jeanfavre S, Avila-Pacheco J, Nakata T, et al. Bacteroides-Derived Sphingolipids Are Critical for Maintaining Intestinal Homeostasis and Symbiosis. *Cell Host Microbe* (2019) 25(5):668–80.e7. doi: 10.1016/j.chom.2019.04.002
33. Vogt NM, Kerby RL, Dill-McFarland KA, Harding SJ, Merluzzi AP, Johnson SC, et al. Gut microbiome alterations in Alzheimer's disease. *Sci Rep* (2017) 7 (1):13537. doi: 10.1038/s41598-017-13601-y
34. Mysak J, Podzimek S, Sommerova P, Lyuya-Mi Y, Bartova J, Janatova T, et al. Porphyromonas gingivalis: major periodontopathic pathogen overview. *J Immunol Res* (2014) 2014:476068. doi: 10.1155/2014/476068
35. Jia L, Han N, Du J, Guo L, Luo Z, Liu Y. Pathogenesis of Important Virulence Factors of Porphyromonas gingivalis via Toll-Like Receptors. *Front Cell Infect Microbiol* (2019) 9:262. doi: 10.3389/fcimb.2019.00262
36. Moye ZD, Valiuskyte K, Dewhirst FE, Nichols FC, Davey ME. Synthesis of Sphingolipids Impacts Survival of Porphyromonas gingivalis and the Presentation of Surface Polysaccharides. *Front Microbiol* (2016) 7:1919. doi: 10.3389/fmicb.2016.01919
37. Dominy SS, Lynch C, Ermini F, Benedyk M, Marczyk A, Konradi A, et al. Porphyromonas gingivalis in Alzheimer's disease brains: Evidence for disease causation and treatment with small-molecule inhibitors. *Sci Adv* (2019) 5(1): eaau3333. doi: 10.1126/sciadv.aau3333
38. Osorio C, Kanukuntla T, Diaz E, Jafri N, Cummings M, Sfera A. The Post-amyloid Era in Alzheimer's Disease: Trust Your Gut Feeling. *Front Aging Neurosci* (2019) 11:143. doi: 10.3389/fnagi.2019.00143
39. De Strooper B, Annaert W. Proteolytic processing and cell biological functions of the amyloid precursor protein. *J Cell Sci* (2000) 113(Pt 11):1857–70.
40. Ishida N, Ishihara Y, Ishida K, Tada H, Funaki-Kato Y, Hagiwara M, et al. Periodontitis induced by bacterial infection exacerbates features of Alzheimer's disease in transgenic mice. *NPJ Aging Mech Dis* (2017) 3:15. doi: 10.1038/s41514-017-0015-x
41. Ilievski V, Zuchowska PK, Green SJ, Toth PT, Ragozzino ME, Le K, et al. Chronic oral application of a periodontal pathogen results in brain inflammation, neurodegeneration and amyloid beta production in wild type mice. *PloS One* (2018) 13(10):e0204941. doi: 10.1371/journal.pone.0204941
42. Wu Z, Ni J, Liu Y, Teeling JL, Takayama F, Collcutt A, et al. Cathepsin B plays a critical role in inducing Alzheimer's disease-like phenotypes following chronic systemic exposure to lipopolysaccharide from Porphyromonas gingivalis in mice. *Brain Behav Immun* (2017) 65:350–61. doi: 10.1016/j.bbi.2017.06.002
43. Tiribuzi R, Orlacchio A, Crispoltoni L, Maiotti M, Zampolini M, De Angeliz M, et al. Lysosomal beta-galactosidase and beta-hexosaminidase activities correlate with clinical stages of dementia associated with Alzheimer's disease and type 2 diabetes mellitus. *J Alzheimers Dis* (2011) 24(4):785–97. doi: 10.3233/JAD-2011-100525
44. Bussian TJ, Aziz A, Meyer CF, Swenson BL, van Deursen JM, Baker DJ. Clearance of senescent glial cells prevents tau-dependent pathology and cognitive decline. *Nature* (2018) 562(7728):578–82. doi: 10.1038/s41586-018-0543-y
45. Musi N, Valentine JM, Sickora KR, Baeuerle E, Thompson CS, Shen Q, et al. Tau protein aggregation is associated with cellular senescence in the brain. *Aging Cell* (2018) 17(6):e12840. doi: 10.1111/acel.12840
46. Zhang P, Kishimoto Y, Grammatikakis I, Gottimukkala K, Cutler RG, Zhang S, et al. Senolytic therapy alleviates Abeta-associated oligodendrocyte progenitor cell senescence and cognitive deficits in an Alzheimer's disease model. *Nat Neurosci* (2019) 22(5):719–28. doi: 10.1038/s41593-019-0372-9
47. Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* (2010) 5:99–118. doi: 10.1146/annurev-pathol-121808-102144
48. Aquino-Martinez R, Rowsey JL, Fraser DG, Eckhardt BA, Khosla S, Farr JN, et al. LPS-induced premature osteocyte senescence: Implications in inflammatory alveolar bone loss and periodontal disease pathogenesis. *Bone* (2020) 132:115220. doi: 10.1016/j.bone.2019.115220
49. Trayssac M, Hannun YA, Obeid LM. Role of sphingolipids in senescence: implication in aging and age-related diseases. *J Clin Invest* (2018) 128 (7):2702–12. doi: 10.1172/JCI97949
50. Lalla R, Donmez G. The role of sirtuins in Alzheimer's disease. *Front Aging Neurosci* (2013) 5:16. doi: 10.3389/fnagi.2013.00016
51. Patel NV, Gordon MN, Connor KE, Good RA, Engelman RW, Mason J, et al. Caloric restriction attenuates Abeta-deposition in Alzheimer transgenic models. *Neurobiol Aging* (2005) 26(7):995–1000. doi: 10.1016/j.neurobiolaging.2004.09.014
52. Qin W, Chachich M, Lane M, Roth G, Bryant M, de Cabo R, et al. Calorie restriction attenuates Alzheimer's disease type brain amyloidosis in Squirrel monkeys (Saimiri sciureus). *J Alzheimers Dis* (2006) 10(4):417–22. doi: 10.3233/JAD-2006-10411
53. Kim D, Nguyen MD, Dobbin MM, Fischer A, Sananbenesi F, Rodgers JT, et al. SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis. *EMBO J* (2007) 26 (13):3169–79. doi: 10.1038/sj.emboj.7601758

54. Julien C, Tremblay C, Emond V, Lebbadi M, Salem N Jr., Bennett DA, et al. Sirtuin 1 reduction parallels the accumulation of tau in Alzheimer disease. *J Neuropathol Exp Neurol* (2009) 68(1):48–58. doi: 10.1097/NEN.0b013e3181922348

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Porphyromonas gingivalis Placental Atopobiosis and Inflammatory Responses in Women With Adverse Pregnancy Outcomes

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The microbiome modulates inflammation at the fetal maternal interface on both term and preterm labor. Inflammophilic oral bacteria, such as *Porphyromonas gingivalis*, as well as urogenital microorganisms (UGM) could translocate to the placenta and activate immune mechanisms in decidual tissue that is associated with adverse pregnancy outcomes (APO). This study establishes the associations between the presence of microbes in the placenta and placental cytokine patterns in women who presented APO, e.g., low birth weight (LBW), preterm premature rupture of membranes (PPROM), preterm birth (PTB) and other clinical signs related to Chorioamnionitis (CA). A total of 40 pregnant women were included in the study and divided into five groups according to placental infection (PI) and APO, as follows: (1) women without PI and without APO (n = 17), (2) women with *P. gingivalis*-related PI and APO (n = 5), (3) women with *P. gingivalis*-related PI and without APO (n = 4), (4) women with PI related to UGM and APO (n = 5) and (5) women without PI with APO (n = 9). Obstetric, clinical periodontal status evaluation, and subgingival plaque sampling were performed at the time of delivery. Placental levels of interleukin IL-1β, IL-6, IL-10, IL-15, IL-17A, IL-17F, IL-21, IL-12p70, tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 α (MCP-1α), granzyme B, and interferon-γ (IFN-γ) were determined using a multiplex flow cytometry assay. All patients showed a predominant Th-1 cytokine profile related to labor, characterized by IFN-γ overexpression. The analysis by groups suggests that Th-1 profile was trending to maintain cytotoxic cell activity by the expression of IL-15 and granzyme B, except for the group with *P. gingivalis*-related PI and APO, which exhibited a reduction of IL-10 and IL-17F cytokines ($p < 0.05$) and a Th-1 profile favoring macrophage activation by MCP-1 production ($p < 0.05$). This study confirms a pro-inflammatory pattern associated with labor, characterized by a Th-1 profile and the activity of cytotoxic cells, which is enhanced by PI with UGM. However, PI associated with *P. gingivalis* suggests a switch where the Th-1 profile favors an inflammatory response mediated by MCP-1 and macrophage activity as a mechanistic explanation of its possible relationship with adverse outcomes in pregnancy.

Keywords: pregnancy outcome, cytokines, periodontitis, dysbiosis, *Porphyromonas gingivalis*, macrophage, inflammation, placenta

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INTRODUCTION

Given the concept that the uterus is a sterile microenvironment, the description of the presence of a healthy microbiome, typical of the placenta, has been widely debated since 2014 by different studies, both in favor (Aagaard et al., 2014; Doyle et al., 2014; Parnell et al., 2017; Seferovic et al., 2019) and against the hypothesis (Leiby et al., 2018; Theis et al., 2019). In fact, those who mentioned the presence of a placental microbiome, characterized its composition as being very similar to the human oral microbiome, i.e., composed of non-pathogenic commensals, such as Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria phyla (Aagaard et al., 2014). However, the role of the microbiome in host response have been explained by the release of metabolic products in adverse pregnancy outcomes (APO), like preterm birth (PTB), low birth weight (LBW), and preterm premature rupture of membranes (PPROM; Parris et al., 2020). It has even been determined that bacterial DNA and short-chain fatty acids produced by commensals in utero have the potential to influence the development of the fetal immune system (Stinson et al., 2019), just as uterine quiescence during pregnancy is mediated by anti-inflammatory mechanisms (Mendelson, 2009).

Nowadays, there is a lack of consensus regarding the definition of the terms chorioamnionitis (CA) or intra-amniotic infection, and a of specific clinical signs and symptoms of intra-amniotic infection even if diagnosed with the gold standard of culture/PCR in amniotic fluid (Morgan, 2016). Nevertheless, statistical analyses of APO associated with intra-amniotic infection by certain microorganisms, have established that intra-amniotic infection may be, not only one of the main risk factors for PTB, but also a direct cause by the force, temporality and the gradient with which the DNA of microorganisms is associated with PTB (DiGiulio et al., 2008).

The abundance of certain species with clinical relevance differs between placentas after spontaneous PTB, non-spontaneous PTB, and term birth and supports the fact that a significant percentage of placentas prone to spontaneous PTB has a component of intrauterine infection (Leon et al., 2018). The frequencies of microorganisms in the intrauterine microenvironment are highly variable, which could be related to the type of samples taken, their processing and the techniques used to detect them or their DNA; these frequencies are also related to the gestation stage at which the APO is presented. The numbers can reach up to 60% in the early stages and fluctuate between 10–25% in the third quarter (DiGiulio et al., 2008; Combs et al., 2014; Morgan, 2016). Specifically, in the placenta, microorganisms can invade the amnion and chorion as well as the villous tree, which is frequently associated with APO including congenital infections (Theis et al., 2019).

The intrauterine microbiome can come from different sites and get there by different routes: it may arrive (1) ascending from the vagina through small channels in the cervical mucus; (2) by the hematogenous route; (3) by transmembrane filtration from the intestine to the peritoneal cavity with retrograde ascension *via* the fallopian tubes; (4) from the intestine or from the blood; or (5) from leukocytes and dendritic cells carrying bacteria and spreading them to other locations such as the uterus (Bardos et al., 2020). The hematogenous route can also be used by microorganisms of urinary and respiratory infections, and from the oral cavity (Cao et al., 2014; Prince et al., 2016).

Periodontitis is an inflammatory non-communicable disease that develops in susceptible subjects from the actions of dysbiotic microbial communities and pathobionts that exhibit synergistic virulence that potentiates the host response, while promoting their own survival by cross feeding from the products of inflammation related to tissue destruction. Periodontitis may eventually lead to tooth loss and systemic complications (Hajishengallis, 2014), including pregnancy complications (Hajishengallis, 2015).

P. gingivalis is considered a keystone pathogen in the pathogenesis of periodontitis. This type of pathogens supports and stabilizes microbiota associated with disease states and has the capacity to cause inflammation even if present in insignificant quantities (Hajishengallis et al., 2012). These bacteria are the most frequent microorganisms present during bacteremia in patients with periodontitis (Lafaurie et al., 2007; Horliana et al., 2014) and they are the most common microorganisms in the amniotic fluid and placental tissue (León et al., 2007; Hasegawa-Nakamura et al., 2011; Vanterpool et al., 2016). This translocation of microorganisms to blood or other tissues has also been described as dysbiosis, so the term atopobiosis has been coined to describe the translocation of microorganisms in order to avoid confusion. Thus, atopobiosis describes the appearance of bacteria at sites other than its usual location and is associated with multiple chronic, non-communicable, and inflammatory diseases (Potgieter et al., 2015).

Whether commensals participate or not in the onset of birth, there is always an inflammatory response that leads to delivery, which includes innate and acquired immune responses; in fact, inflammatory responses are part of pregnancy itself. During pregnancy estrogen and progesterone favor the humoral immune response; estrogens stimulate the production of antibodies and the activation of natural killer (NK) cells and macrophages and they decrease the production of proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α). Progesterone may inhibit lymphocyte activation and reduce Th-1 profile cytokines [IL-1β, IL-2, IL-12, IL-15, IL-18, interferon-γ (IFN)-γ, and TNF-α], so an increase in the levels of these hormones may be involved in maternal-fetal nonrejection (Piccinni et al., 2000). However, at the end of the third trimester, a pattern of Th-1-type cytokines predominates since an inflammatory environment is necessary for the onset of labor (Saito et al., 2010).

The presence of several cytokines in the amniotic fluid has been reported as an inflammatory marker to predict the risk of preterm delivery (Liu et al., 2017a). Th-1-type cytokines (IFN-γ, IL-12p70, IL-15, and IL-18) can be over-regulated by infection and proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-21 may participate in the adverse outcomes of pregnancy (Erlebacher, 2013). Another less studied mechanism is related to the abnormal regulation of NK cells by natural cytotoxicity receptors, which regulate NK cell cytotoxicity and cytokine production or the activation of decidual macrophages

by MCP-1 and other profiles of cytokines such as the Th-17 profile, which have been implicated in APO such as PTB and PPROM (Fukui et al., 2009; Bardou et al., 2014; Liu et al., 2017b).

A complex interaction between infection and inflammation on both the systemic and intrauterine environments and various biological processes between pathogen and host seem to be crucially involved in the pregnancy outcomes. However, the mechanisms of the immune activation pathways and the triggers of the immune response at the molecular level, associated with induction of preterm labor are not clear. Causal infectious agents, the role of polymicrobial infections, critical sites of infection, as well as the participation of cells and activation pathways of the immune response have barely been identified (Cappelletti et al., 2016).

Chopra et al. (2020) based on the evidence, suggest the atopobiosis of *P. gingivalis* to the amniotic fluid and placenta of women with APO; however, the molecular mechanisms by which *P. gingivalis* relates to the occurrence of complications during pregnancy and in the newborn are not clear in the literature (Chopra et al., 2020). Some of the potential pathogenic mechanisms which might link *P. gingivalis* and APO include: (1) Direct invasion, translocation, and injury to the foetal-placental unit/interface and maternal tissues, (2) Persistence and survival within the foetal and maternal tissues and immune response evasion, (3) Increased production of proinflammatory cytokines and shift in maternal-foetal immune response from Th2 to Th1 with the onset of Th17/T regulatory cell imbalance, (4) Activation of the acute-phase response, (5) Onset of Polymicrobial dysbiosis and development of pathobiont species, (6) Increased oxidative stress in the foetal and maternal tissue, and (7) Increased foetal adrenal cortisone production and the onset of foetal stress (Chopra et al., 2020).

Based on the occurrence of APO and the presence or absence of placental infection (PI), this study seeks to establish a possible relationship between the presence of PI by *P. gingivalis* and urogenital microorganisms (UGM) and the pattern of cytokine expression in the placenta (IFN-γ, IL-12p70, IL-17A, IL-17F, IL-23, IL-21, IL-10, IL-15, granzyme B, MCP-1, TNF-α, IL-1β, and IL-6) which may be related to the occurrence of APOs (e.g., LBW, PPROM, and PTB) and other clinical signs related to CA.

MATERIALS AND METHODS

This study involved a group of subjects from a larger previously published case control study (Montenegro et al., 2019) whose objective was to stablish the association between oral and UGM in the placenta and PTB, PPROM, and clinical signs of intraamniotic infection. All participants agreed to participate in the study and signed an informed consent approved by the Institutional Ethics Committee of Universidad El Bosque, PCI-2014-18.

Study Population

A total of 224 pregnant women aged ≥18 years that were admitted for delivery at the Service of Gynecology and Obstetrics of Hospital Simon Bolivar in Bogotá, Colombia from August 2014 to August 2016 were initially enrolled in the case control study of oral and urogenital intra-amniotic infection in women with preterm delivery (Montenegro et al., 2019), in which women included should have been monitored in the hospital during their pregnancy and have a detailed record of prenatal care, especially records for the presence or absence of associated infection, vaginosis, and CA. The diagnosis of clinical CA was based on the Gibbs and Duff antepartum criteria and on the clinical criteria of the abnormal appearance of post-delivery membranes postpartum. All of the patients that received attention at the emergency department of Simon Bolivar Hospital provided their relevant medical history, where symptoms of a potential PTB or intra-amniotic infection were identified (i.e., uterine activity, uterine hypersensitivity, expulsion of mucus plug and/or poor genital bleeding, maternal or fetal tachycardia, and fever). Women with antibiotic intake 1 month before delivery, uterine abnormalities, fetal malformations, twin pregnancy, heavy vaginal bleeding, fetal distress, infectious or systemic diseases such as HIV, tuberculosis, candidiasis or diabetes, smokers or users of alcohol or psychoactive substances, and women with premature termination of pregnancy for medical reasons were excluded. If the placental sample was contaminated in the operating room during delivery or the sample could not be analyzed due to its quantity, the woman was not included; six women were excluded for these reasons.

For cytokine analysis, a second phase was carried out and the patients were selected based on the results of first phase specially the presence of infection and perinatal condition. In order to control the influence of age and other reasons of inflammation, all women older than 40 years and patients with pre-eclampsia were excluded.

Figure 1 shows a flowchart describing the reference population, selection and exclusion of participants to establish the cytokine analysis groups according to APO and placental UGM/*P. gingivalis* infection. The groups were distributed as follows:

Group 1 – Control group for APO and PI. All women without APO, with a suitable pregnancy control (≥ 6 prenatal controls) and periodontal health with very low rates of inflammation evidence (<15% of gingival bleeding) $n = 17$.

Group 2 – *P. gingivalis*-related PI with APO. All women with PI associated with *P. gingivalis* with APO (one aged >40 years was excluded) $n = 5$.

Group 3 – *P. gingivalis*-related PI without APO. All women with PI associated with *P. gingivalis* without APO (two samples could not be analyzed due to its quantity) $n = 4$.

Group 4 – UGM-related PI with APO. All women with PI associated with UGM with APO (three participants were excluded due to preeclampsia or >40 years old) $n = 5$.

Group 5 – No PI with APO. nine women with APO without PI paired by age with nine women with APO/PI including infection by *P. gingivalis* and by UGM only. $n = 9$ (control group for APO with placental infection).

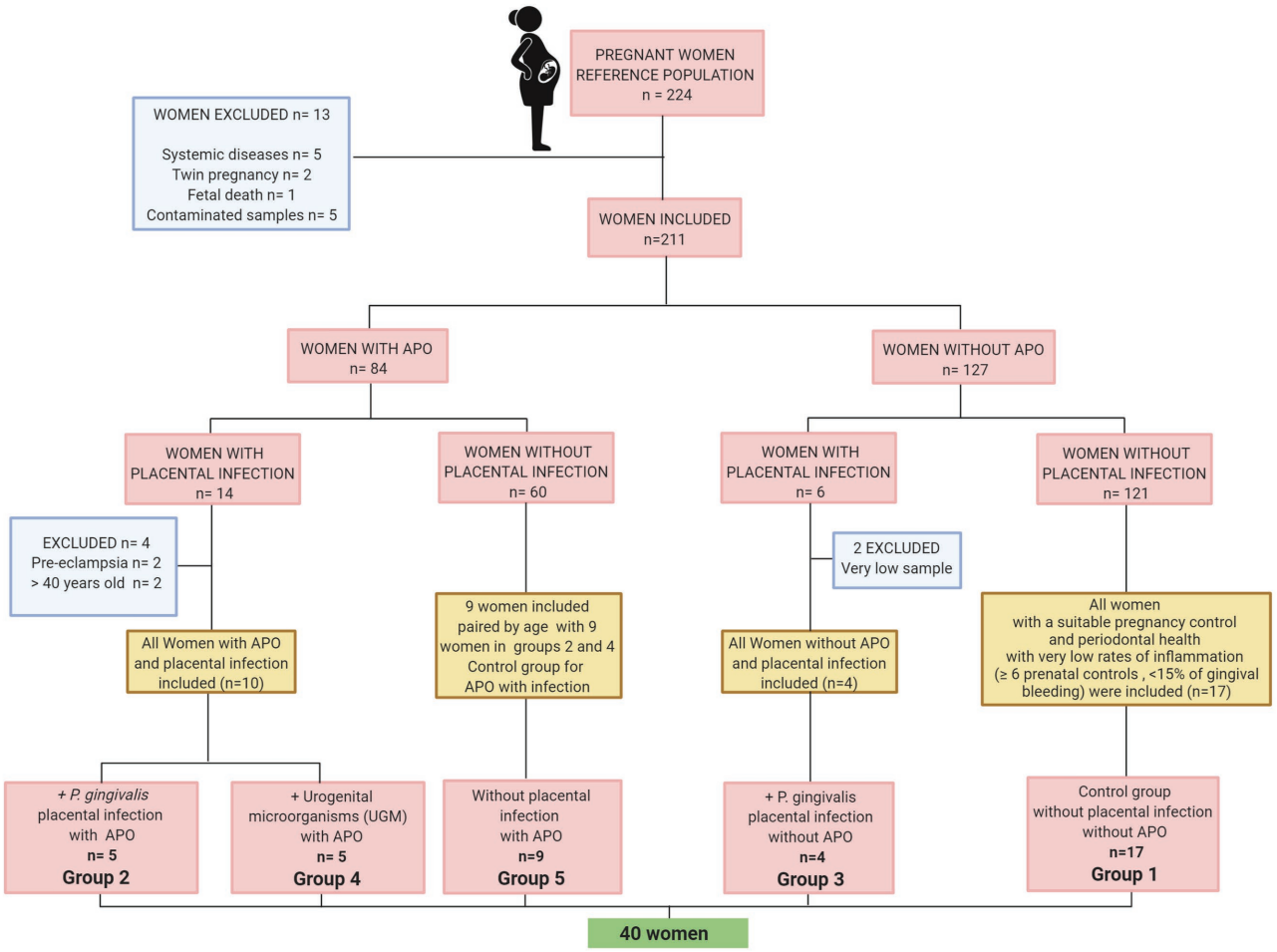

FIGURE 1 | Flow chart of sample selection.

Adverse Pregnancy Outcome Assessment

PTB was established for those births that occurred before 37 weeks of gestation, and LBW was established for infants weighing <2,500 g. PPROM was established for those patients who had >12 h from rupture to delivery. Clinical signs of PI were identified as fever, maternal tachycardia, fetal tachycardia, and fetid fluid (Gibbs and Duff, 1991).

Periodontal Clinical Examination and Subgingival Plaque Sampling

Two calibrated periodontists (properly trained before the beginning of the study), who were blinded regarding the group category at the time of the evaluation, performed the periodontal assessment of all patients before or up to 8 h after delivery. A full-mouth examination was performed at six sites on each permanent tooth using a North Carolina® probe (PCPUNC-15; Hu-Friedy, Chicago, IL, United States). The indices employed for the diagnosis were: probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP), plaque index, and gingival index (GI). The presence of periodontitis was determined according to the criteria established by the Centers for Disease Control and Prevention/ American Academy of Periodontology (≥ 2 interproximal sites with CAL ≥ 3 mm and ≥ 2 interproximal sites with PD ≥ 4 mm; not on the same tooth; Eke et al., 2010).

The supragingival plaque was removed, and absorbent paper points were inserted for 20 s into the six deepest periodontal sites of each subject for polymerase chain reaction (PCR) detection of *P. gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Eubacterium nodatum*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* in the subgingival plaque.

Placental Tissues Sample Collection

After delivery, two samples of amniotic membrane (1.5 cm^2) were taken: one at the umbilical cord insertion base and the second at the free edge of the contralateral cotyledon of the first sample, which were transferred to an empty sterile microcentrifuge tube and refrigerated at −20°C until processing. One sample was used for extraction and amplification of bacterial DNA in the placenta and the other one for tissue cytokine profile detection in patients with PI.

Extraction and Amplification of Bacterial DNA in the Placenta

The disintegration of the placenta samples was performed until total tissue trituration was achieved; each sample was weighed in triplicate under sterile conditions and deposited in a 1.5 ml microcentrifuge tube and 80 µl phosphate-buffered saline (PBS) was added to homogenize in vortex. All tubes with the samples were submerged in liquid nitrogen for 5 min to achieve a complete lysis of the tissue. After thawing, 100 µl buffer ATL, 3 µl proteinase K, and 16 µl lysozyme were added and mixed in vortex until complete homogenization was achieved. The samples were incubated with shaking at a temperature of 56°C for 24 h until complete lysis was achieved and vortexed again.

To extract DNA, the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used. Before DNA amplification, the purity and amount of DNA were obtained using NanoDrop 2000 (Thermo Scientific, Rockford, IL, United States). Bacterial DNA amplification was performed by nested PCR using a universal primer for Eubacteria for the first amplification EUB27F: 5'-GAG TTT GAT CCT GGC TCA G-3' and EUB1544R: 5'-AGA AAG GAG GTG ATC CAG CC-3', directed to the amplification of a fragment of the 16S rRNA gene, common among the bacteria. The amplification was evidenced by the presence of the amplification product (1407 bp) in agarose gel (2%) with 0.5 µg/ml ethidium bromide. The amplification of the specified DNA for each microorganism was done using the PCR product obtained from the amplification as the template for the universal primer of oral bacteria such as *P. gingivalis*, *T. forsythia*, *T. denticola*, *A. actinomycetemcomitans*, *Campylobacter rectus*, and *Prevotella intermedia* (Ashimoto et al., 1996), *Parvimonas micra*, and *F. nucleatum* (Kuriyama et al., 2000), and the microorganisms associated with urogenital infection such as *Gardnerella vaginalis* (Fredricks et al., 2007), *Ureaplasma urealyticum* (Teng et al., 1994), *Mycoplasma hominis* (Blanchard et al., 1993), and *Candida albicans* (Mahmoundi-Rad et al., 2012). Those included in the DNA of reference strains of the American Type Culture Collection (Manassas, VA, United States) were used as positive controls and sterile water was used as negative control. The identification of each microorganism was confirmed by the presence of amplification products using agarose gel electrophoresis (1.5%) and 0.5 µg/ml ethidium bromide staining.

Cytokine Profile Analysis

Dissection of Placenta and Tissue Cytokine Profiles Detection

The placental samples were homogenized by mechanical disaggregation of tissues using the Medimachine System (120V; BD™, San Jose, CA, United States). The samples were cut in small pieces, which were inserted into a Medicon of 35 µm (BD™ Medimachine Medicon, Sterile) by adding 10 µl protease-free PBS (Sigma, St. Louis, MO, United States) for 3 cycles at 80 rpm. The homogenized samples were passed through Filcons of 20 µm (BD™ Medimachine Filcon, Sterile, Cup-Type), a washing cycle at 1800 rpm for 5 min was done, and the supernatants were recovered to measure the soluble cytokine levels using a multiplex bead-based LEGENDplex™ assay by flow cytometry. A Human Th Cytokine mix and match 13 plex Panel (San Diego, CA, United States) was customized, in which 13 capture beads of two different sizes were used, each one conjugated with antibodies against IFN-γ, IL-12p70, IL-17A, IL-17F, IL-23, IL-21, IL-10, IL-15, granzyme B, MCP-1, TNF-α, IL-1ß, or IL-6. The samples were processed according to manufacture instructions in a sandwich immunoassay. The flow cytometry acquisition was carried out in a BD FACS Accuri™ C6 Plus using the BD Accuri™ C6 Plus software; the analysis was performed based on a standard curve into a logistic regression model (five points; BioLegend's LEGENDplex™ Software, 2016). The results were expressed in pg/ml units.

Statistical Analysis

In order to find correlations among cytokine profile groups and APO, a Factorial analysis of mixed data (FAMD) was performed that combined Principal Component Analysis (PCA) for continuous variables and Multiple Correspondence Analysis (MCA) for categorical variables (Audigier et al., 2016). Kruskal-Wallis/Mann-Whitney U test was used to compare the cytokines between the different groups. One-way ANOVA with Dunnett's *post-hoc* test was performed to compare the relationship between cytokine/IFN-γ among groups. Spearman rank based pairwise correlation analysis was performed to analyze cytokine abundances in all the groups. R statistical software and IBM-SPSS version 23 were used for all analyses. Heatmaps were made using Morpheus, https://software.broadinstitute.org/morpheus.

RESULTS

Sociodemographic Characteristics of the Study Population

In total 40 patients were included to assess the cytokine response and the association with atopobiosis of *P. gingivalis* to placental tissue (**Figure 1**). The sociodemographic analysis shows homogeneity among groups (**Table 1**). There were no significant differences in age, marital status, and schooling between groups ($p > 0.05$).

Periodontal Clinical Characteristics and Identification of Microorganisms in Subgingival Plaque

Oral clinical assessment showed periodontitis presence in group 2 (*P. gingivalis*-related PI with APO), group 3 (*P. gingivalis*-related PI without APO) and group 4 (UGM-related PI with APO; **Table 1**), all of them associated with *P. gingivalis* presence in subgingival plaque (60, 75, and 40% respectively; **Supplementary Table 1**). In Patients with subgingival presence of *P. gingivalis* higher indices were more frequent and the highest median was observed in the *P. gingivalis*-related PI without APO (group 3) which presented a higher frequency of periodontitis ($p < 0.05$). No significant differences were observed for plaque index, PD, or CAL among groups

TABLE 1 | Socio-demographic characteristics and periodontal condition according to the presence of placental infection (PI) and adverse pregnancy outcomes (APO).

	Control	*Pg* + APO+	*Pg* + APO-	UGM + APO+	PI- APO+
	n = 17	*n* = 5	*n* = 4	*n* = 5	*n* = 9
Age mean ± SD	21.29 ± 3.1	24.60 ± 4.6	19.75 ± 1.7	22.20 ± 3	19.33 ± 1.2
Civil status (F%)					
Single	5 (29.4)	0	2 (50)	1 (20)	2 (22.2)
Married	1 (5.9)	0	0	0	1 (11.1)
Civil union	11 (64.7)	5 (100)	2 (50)	4 (80)	6 (66.7)
Education (F%)					
Elementary	0	0	0	0	2 (22.2)
High school	15 (88.2)	4 (80)	4 (100)	5 (100)	6 (66.7)
Technical	2 (11.8)	1 (20.0)	0	0	1 (11.1)
Periodontitis n (%)					
Yes**	0 (0.0)	3 (60.0)	3 (75.0)	2 (40.0)	0 (0.0)
Clinical signs of placental infection (F%)					
Yes	0 (0.0)	2 (40.0)	0 (0.0)	0 (0.0)	0 (0.0)

***p < 0.05 statistically significant difference among groups by Chi square test. Clinical signs of infection included uterine hypersensitivity, fever, maternal tachycardia, fetal tachycardia, and fetid flow. APO, adverse pregnancy outcome; UGM, urogenital microorganisms; SD, standard deviation; IQR, Interquartile range; Pg+ APO+, P. gingivalis (+) adverse pregnancy outcome (+); Pg+ APO–, P. gingivalis (+) adverse pregnancy outcome –; UGM+ APO+, urogenital microorganisms (+) adverse pregnancy outcome +; PI– APO+, Placental infection (–) adverse pregnancy outcome (+); Control, adverse pregnancy outcome (–) without placental infection.*

($p > 0.05$). Distribution of microorganisms in subgingival plaque is presented in **Supplementary Table 1**.

Factorial Analysis of Mixed Data for Cytokine and Adverse Outcomes

Figure 2 shows the correlation between both quantitative and qualitative variables, and their contributions in two dimensions: 1 (without APO) and 2 (with APO). The contribution of the variables for delivery without APO show a pattern of cytokine expression predominantly inclined toward higher correlation among IFN-γ and NK related cytokines and secondary cytokines of Th17 profile, in contrast to the adverse outcomes dimension in which the correlations reveal that inflammatory cytokines associated with macrophage activation such as MCP-1, IL-6, and IL-1β are strongly correlated with the group variables (**Figures 2A,B**). To establish which group contributed to this correlation in Dim-2 with MCP-1, IL-6, and IL-1β, the FAMD allowed us to make an extraction by categories together with their contributions and correlations, showing that macrophage cytokines were mainly related to the *P. gingivalis*-related PI with APO (group 2), which was the group with the greatest contribution to the model representing a differential pattern associated to an activation of inflammatory response (**Figure 2C**).

Analysis of Cytokine Profiles Between the Groups With or Without PI

Based on the previous results in which all profiles showed a similar behavior among the groups, except for the *P. gingivalis*-related PI with APO group, cytokine levels were analyzed according to Th-1, Treg and Th-17 profiles and compared by groups. As **Figure 3** shows, this analysis allowed us to observe a generalized significant decrease in levels of the Th-1 (IFN-γ, IL-12p70, IL-15, Granzyme-B, TNF-α, and IL-1ß), Th-17 (IL-17F, IL-17A, IL-21, and IL-23), and Treg (IL-10) profile cytokines in the *P. gingivalis*-related PI with APO group when compared with the controls ($p < 0.05$). Meanwhile, macrophage activation cytokines (MCP-1 and IL6) in Th-1 profiling were maintained in both groups (*P. gingivalis*-related PI with APO and the controls), suggesting an important role for these cells in APO related to *P. gingivalis* placental infection. UGM infections did not show significant differences with the control group for any cytokine profile (**Figure 3**). **Figure 4** shows an analysis of correlations between host cytokine levels in control, *P. gingivalis-related* PI with APO (Pg + APO+), *P. gingivalis*-related PI without APO (Pg-APO+), UGM-related PI with APO (UGM + APO+), and No PI with APO (PI-APO+) groups by Spearman's rank-based correlations (**Figures 4A**–**E**). All groups showed high positive correlations (>0.6) between IFN-γ with IL-1β, IL-15, IL-17F, IL-21, IL-12p70, TNF-α IL-10, granzyme-B, and IL-17A but low correlations with MCP-1 and IL6. However, a high but negative correlation (> − 0.6) was observed in Pg + APO+ group between IFN-γ and MCP-1, IL-1β, and IL-6 levels. *P. gingivalis*-related PI with APO group showed also a negative correlation between IL-21, IL-17F with MCP-1, IL-1β, and IL6. High and negative correlations were found between all cytokines with IL6 in *P. gingivalis*-related infection with APO group but low correlations between all cytokines with MCP-1 and IL-1β.

Cytokine/IFN-γ Ratio in Placental Tissue

Results showed that at the time of delivery the predominant profile was Th-1 in all of the groups. To identify the cell line in which that profile was polarized, the expression ratios for each cytokine were determined relative to the concentration of IFN-γ and were compared between groups, showing a common behavior of the activation of NK cells as the main target of Th-1 response in childbirth. *P. gingivalis*-related PI with APO group showed a variation in the response pattern, where MCP-1/IFN-γ, TNF-α/IFN-γ, and IL-21/IFN-γ

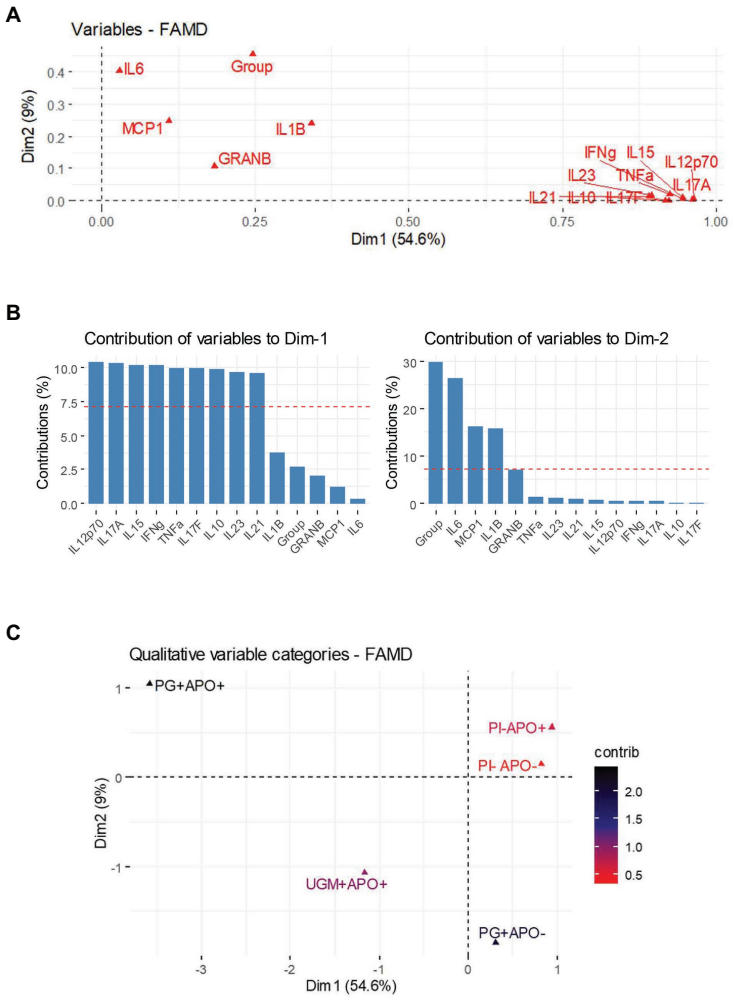

FIGURE 2 | Factorial analysis of mixed data (FAMD) for cytokine and adverse outcomes. FAMD combines Principal Component Analysis (PCA) for continuous variables and Multiple Correspondence Analysis (MCA) for categorical variables in at least two dimensions. In this case Dim-1 represents those deliveries without APO and Dim-2 represents deliveries associated with APO. **(A)** shows a variables vector map as a geometric representation of variables presented by arrows where the length expresses the SD of each variable, and the angles among variables show their correlations (acute angles represent stronger correlations, right angles no correlations and straight/obtuse angles show inverse correlations). Note how cytokines from Th-1 [polarized to natural killer (NK) cell activation], Th-17 and T-reg are grouped in a network of acute angles showing a common behavior on Dim-1. However, cytokines associated with macrophage activation are leaning next to Dim-2 and near group variable. **(B)** shows a bar chart with the contribution of each cytokine to each dimension; the percentage could be understood as the weight of this variable in the dimension. In this way Dim-1 is dominated by Th-1 (polarized to NK cell activation) and Th-17 cytokines, while Dim-2 is polarized toward macrophage activation cytokines and group variable. The graphical representation of the components and the heat chart in **(C)** represent the final step in FAMD, showing an extraction to distinguish which group determines the principal contribution to each dimension. The results show a remarkable contribution of *P. gingivalis* placental infection and APO group to Dim-2.

showed differences with significantly higher concentrations (MCP-1/IFN-γ, $p = 0.022$; TNF-α/IFN-γ, $p = 0.004$; and IL-21/IFN-γ, $p = 0.0001$; **Figure 5**).

These results for *P. gingivalis*-related PI with APO group reflects high levels of IFN-γ with a significative decreasing of NK-related cytokines ($p < 0.05$) and the maintenance of those which are associated to macrophage activation when it was compared with the control group. In contrast, the other groups evaluated in this study presented a very similar pattern of cytokine expression characterized by high levels of IFN-γ and NK-related cytokines.

DISCUSSION

It is well known that during pregnancy there are multiple factors (hormonal, metabolic and immunological) that impact and influence the oral microflora; nevertheless, the role of oral atopobiosis in modulating inflammation in conditions like pregnancy is not yet clear since the oral microflora during gestation is still poorly characterized. This is even more relevant if the similarities of the placenta microbiome and that of the oral cavity are considered, which highlights the importance of atopobiosis of oral microorganisms in APOs (Balan et al., 2018).

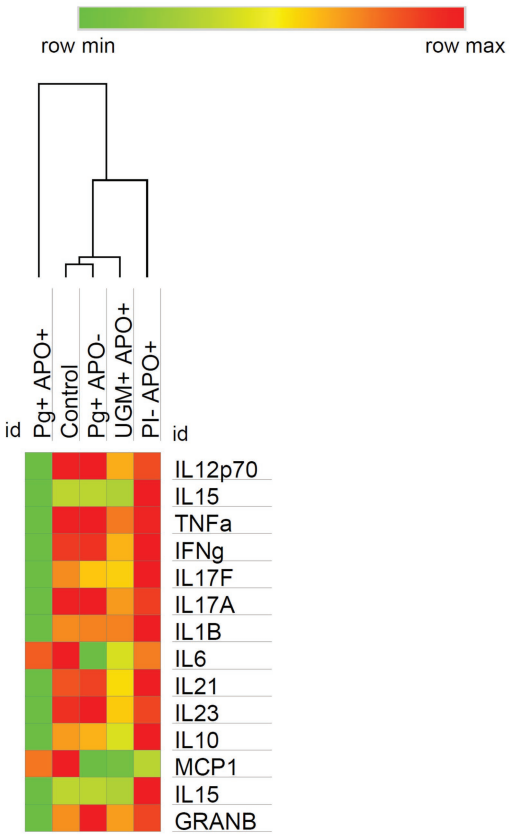

FIGURE 3 | Cytokine profiles among groups analysis. The heat map shows the soluble cytokine concentrations in the controls and their variations among groups at time of delivery. Observe how the behavior in the profiling expression is similar in all groups except in the *P. gingivalis* PI with APO group which reflects a switch in two ways: (1) A significative decrease in the levels of cytokines associated with Th-1 (polarized to NK cell activation), Th-17 and T-reg, and (2) A maintenance of macrophage cytokine levels in contrast with the control group. It should be noted that in all cases the cytokine with higher levels of expression was IFN-γ which suggests the predominance of Th1 profile. Comparisons among groups were made by Kruskal-Wallis and Mann-Whitney U test with a confidence level of 95%. https://software.broadinstitute.org/morpheus.

The amniotic cavity of women with PTB has a much higher DNA content (more than previously believed) with a great diversity of microorganisms, including many that have not yet been characterized; The strength of these findings, due to the amount and timing in which they occur, could be useful in formulating a hypothesis to establish a causal association with APOs (DiGiulio et al., 2008); In the study from which our samples came from (Montenegro et al., 2019), the percentage of intrauterine infection was 9.47% (20/211), similar to that reported by previous studies, in which percentages varied between 10 and 25% in the third trimester of pregnancy (DiGiulio et al., 2008; Combs et al., 2014; Morgan, 2016).

P. gingivalis was the most prevalent identified oral microorganism in PI (12/211, 5.68%); however, in most cases it was not associated with the presence of clinical signs of intrauterine infection, although its frequency was higher in cases with APO (Montenegro et al., 2019). In the *P. gingivalis* PI cases, the presence of periodontitis linked to the subgingival presence of *P. gingivalis* was greater (60% for group 2 and 75% for group 3) and independent of the presence of APO. In group 4, where there was no PI due to *P. gingivalis*, the prevalence of periodontitis was lower and in the group with aseptic APOs, there was no periodontitis. Thus, not only the presence of the bacteria determines the occurrence of conditions or diseases linked to it. This would explain why not all the subjects with subgingival *P. gingivalis* have periodontitis or why not all the patients with PI with *P. gingivalis* show signs of clinical intrauterine infection or APO. Other factors, such as the amount of inoculum, virulence of the isolated microorganism, and individual immune response to the microorganism, could be associated with perinatal complications (Montenegro et al., 2019). *P. gingivalis* itself is not a proinflammatory pathogen, but by using different mechanisms it evades and alters components of the host's immune response (disrupts innate immunity: TLRs and complement), which alters the development of the entire biofilm; then *P. gingivalis* exerts its role of "keystone pathogen" modulating the host response in susceptible individuals (Hajishengallis et al., 2012), which could explain why findings

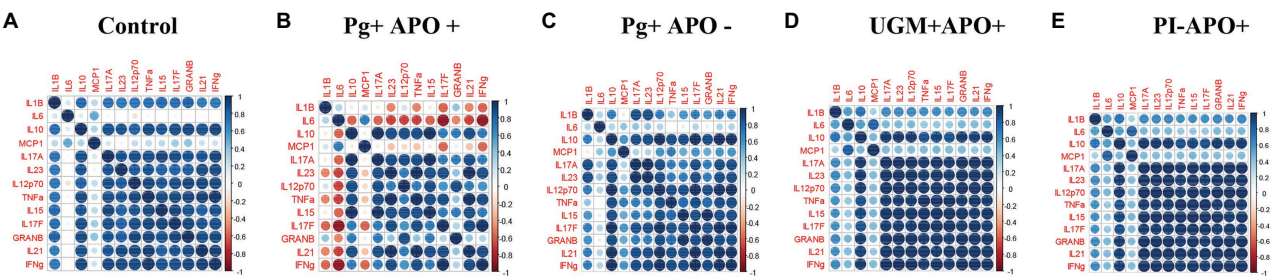

FIGURE 4 | Correlation analysis between cytokines. Spearman rank based pairwise correlation analysis among cytokines (IL-1β, IL-6, IL-10, IL-15, IL-17A, IL-17F, IL-21, IL-12p70, TNF-α, MCP-1, granzyme-B, and IFN-γ) in **(A)** control, **(B)**, Pg + APO + **(C)** Pg + APO - **(D)** UGM + APO+ **(E)** PI-APO+. The size of the spheres represents *p*-value. While strong correlations are shown by large circles, weak correlations are shown by small circles. The color of the circle denotes the strength of the correlation. Perfect positive correlation (with correlation coefficient 1) is indicated in dark blue, whereas perfect inverse correlation (with correlation coefficient 1) are colored in dark red.

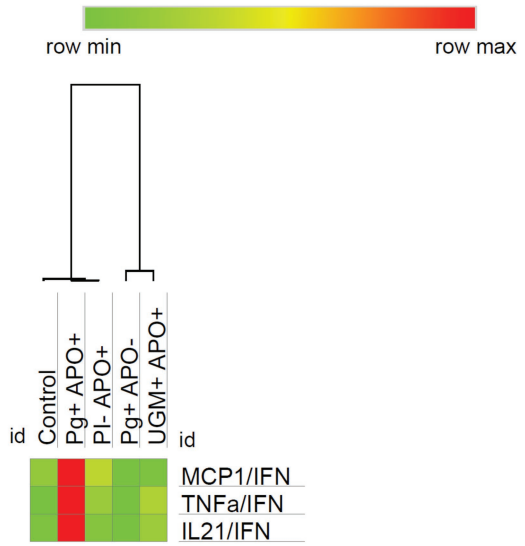

FIGURE 5 | Relationship of MCP-1, TNF-α and IL-21 with IFN- γ. To identify the cell line polarized by Th-1 profile, the expression ratios for each cytokine were determined relative to the concentration of IFN-γ and were then compared between groups. As the heat map shows the *P. gingivalis* PI with APO group presents a particular profiling characterized by macrophage activation mediated *via* MCP-1/IFN-γ ($p = 0.022$), TNF-α/IFN-γ ($p = 0.004$), and IL-21/IFN-γ ($p = 0.0001$). Comparisons were done by one-way ANOVA with Dunnett's post-hoc test with a confidence level of 95%. https://software.broadinstitute.org/morpheus.

of *P. gingivalis* PI may not be always linked to the occurrence of APO (**Supplementary Table 2**).

Some studies suggest that intrauterine tissues in women with PTB or TB may have bacteria present without overt infection (Stout et al., 2013), but commensal bacteria can convert macrophages from the decidua to a proinflammatory state triggering labor. In other words, commensals present in the decidua trigger protease amplification of the inflammatory response by activating the resident macrophages (autocrine action) as well as other leukocytes, which would lead to signaling pathways being activated, resulting in the expression of genes from inflammation that starts labor. This raises the question of whether vaginal bacteria could be the initiators of term and preterm labor. Effaces of the cervix and its dilation can lead to the fetal membranes being exposed to the vaginal microbiome, which provides an entry route to the intrauterine microenvironment where they can initiate an inflammatory response that starts labor (Walsh et al., 2020).

The group with *P. gingivalis*-related PI with APO (group 2) showed clinical signs of intrauterine infection, which could indicate alterations in the mechanisms of inflammation in subjects susceptible to both infection and inflammation. There are multiple mechanisms described by which *P. gingivalis* could be related to the above. In the murine model, changes in gestational tissues have been observed after translocation of *P. gingivalis*, including areas of focused necrosis and increased inflammatory infiltrate. An increase in the expression of Fas, Fas-L and TLR2 at the placental level related to PTB is also observed in rats (Liang et al., 2018). On the other hand, the virulence of microorganisms in the placenta seems to depend on other factors, including genotype, bacterial load, host physiology, and environmental factors (Katz et al., 2009; Gomez-Arango et al., 2017; Fischer et al., 2019). Changes in the microbiome that are accompanied by significant variations in the microbial metabolic pathways as determined in integrative metagenomics analyses, can also contribute to the risk of PTB with or without severe CA (Prince et al., 2016).

Other bacterial species have also been identified in adverse pregnancy events. In this study, we identified PI by bacterial species of urogenital origin, such *M. hominis* and *C. albicans* in women with APO. *Mycoplasma hominis* and *Candida albicans* have been closely associated with APO such as PPROM, PTB, and LBW (Allen-Daniels et al., 2015; Farr et al., 2015). Infections by these microorganisms induced an immune response characterized by a significant increase in Th-1 proinflammatory profiles and NK cell activation. The inflammatory response mediated by IL-1β, TNF-α, PAF, IFN-γ, IL-6, PGE2, MMP-1, and MMP-9 causes the rupture of fetal membrane, increasing uterine contractions (Helmo et al., 2018). Meanwhile, women with *P. gingivalis* PI and APO showed a differential immune response characterized by macrophage activation that could explain the behavior of this infection in preterm delivery.

Although infection is a major risk factor for APO, it is possible that some outcomes occur in the absence of bacteria. In this study, in group 5 where the APO was aseptic, there was also no presence of periodontitis or presence in the subgingival plaque of the studied microorganisms. Actually, it has been reported that intra amniotic inflammation per se is associated with APO even in the absence of microorganisms; intrauterine colonization in the absence of inflammation appears to be relatively benign, but the presence of infection also seems to be related to the presence of inflammation, especially to increased levels of IL-6 that are related to increasing numbers of bacteria (Combs et al., 2014). On the other hand, most of these infections are subclinical and their diagnosis and treatment are difficult during the clinical course of the pregnancy (McCormick, 1985).

The analysis of the role of microorganisms in the host response at the uterine level in PTB, it has been stated that microorganisms in the uterus could increase the synthesis and release of proinflammatory cytokines, prostaglandins, and metalloproteinase, leading to cervical maturation, PPROM, uterine contractions, and finally PTB (Lannon et al., 2014). Normal pregnancy is marked by a state of increased inflammation throughout the gestation period that ends with the inflammatory cascade that begins labor. At conception, maternal immunity changes from a Th-1 dominated-proinflammatory state to a Th-2 immuno-tolerant response that allows fetal implantation and growth (Piccinni et al., 2000). A dysregulation of maternal immunity can lead to a placental invasion and a restriction of fetal growth leading to PTB, preeclampsia, and LBW. Although acute gestational stimuli such as infections are risk factors for PTB and LBW (Park et al., 2018), recent studies have shown that endogenous immune processes such as the presence of chronic inflammation can influence APO in the

absence of infection (Ragsdale et al., 2019). At the end of the third trimester, a Th-1 pattern predominates, and some authors have considered that this change to an inflammatory environment is necessary to start delivery (Saito et al., 2010), as is corroborated by the results of this research. Thus, the present results support the cascade of events described in the literature for the occurrence of labor and we think that it can be precipitated by trigger events (e.g., infection, inflammation caused or not caused by microorganisms, stress, etc.); They are: (1) Activation of innate and adaptive immune cells with increased migratory activity, (2) Recruitment to the fetal maternal interface of activated cells mediated by the release of chemokines; and (3) Amplification of the inflammatory response by infiltrating leukocytes (Gomez- Lopez et al., 2014).

Additionally, in this study, IL-10 showed the lowest concentration in *P. gingivalis* PI groups. The decrease of IL-10 concentration is an important factor in the control of local inflammatory states associated with premature labor and CA because the reduction of this cytokine during the final stages of pregnancy with the presence of a pro- inflammatory state can induce PTB (Hanna et al., 2006; Azizieh and Raghupathy, 2017). However, other authors have established that the overregulation of this cytokine in the presence of infection, as another mechanism, can induce the suppression of NK and LT, thus determining the outcome of pregnancy (Arenas-Hernandez et al., 2015).

After analyzing the profiles of the different cell populations, the response identified in this study supports a macrophage activation pattern promoting an inflammatory activation with a failed regulatory mechanism which could explain the adverse outcomes identified in it. Lipopolysaccharides of Gram-negative bacteria induce the expression of MCP-1, wherein high levels are important in the development of PRM, PTB, and LBW with the presence of PI (Esplin et al., 2005). Different studies suggested that IFN-γ is primordial in the activation of macrophages by the induction of MCP-1 during labor and in the presence of APO in pregnancy (Jacobsson et al., 2003; Esplin et al., 2005). Studies in animal placenta have established that *P. gingivalis* infection also increases macrophages in placental tissue with increased local expression of mediators such as TNF-α and COX-2 (Arce et al., 2012).

The role of macrophages in childbirth is well documented; Their participation occurs in multiple ways that include: Participation in the remodeling of the cervix at delivery due to MMP secretion (such as MMP9) both in PPT and at term birth, related to an increase in macrophages residing in the decidua at the moment of delivery (Gomez-Lopez et al., 2014); It could be supposed that the presence of microorganisms (e.g., *P. gingivalis*) is related to a dysregulation of the system by activation of the previously described proinflammatory mechanisms, which could trigger labor prematurely.

Furthermore, the lipopolysaccharides of *P. gingivalis* induced a significant increase in the expression of COX-2, IL-8, and TNF-α in the trophoblast HTR-8 through the TLR-2/TLR-4-NF-κB signaling pathway, supporting the potential of *P. gingivalis* to induce proinflammatory effects in the placenta (Arce et al., 2012; Ao et al., 2015). The increase in MCP-1, TNF-α, and IL-21 and the decrease of IFN-γ for the *P. gingivalis* PI and with APO group suggest that *P. gingivalis* would directly activate the production of MCP-1 without having to be induced by high concentrations of IFN-γ. Likewise, IL-21 as an independent worker of Th-17 profile might be directly induced by bacteria lipopolysaccharides to modulate the activity of macrophages (Li et al., 2013). On the other hand, enhanced innate cell responses to *P. gingivalis* lipopolysaccharides with a decreased peripheral NK cell function in patients showing a relative NK cell energy have been described, which may be implicated in the pathogenesis of systemic illnesses that are linked to periodontitis (Gaudilliere et al., 2019).

Another immunological mechanism that has been proposed to explain preterm delivery corresponds to detectable levels of cytokines associated with the Th-17 profile that has been reported to be related to the degradation of the decidua and that increase in cases of PTB with CA (Saito et al., 2010). However, in the present study, these cytokines showed a reduction in the *P. gingivalis* PI with adverse event group (group 2), which could indicate that the cytokines of the Th-17 profile do not seem to be increased or related to the adverse event with PI associated with *P. gingivalis*. In contrast, it was observed that IL-17F regulating IL-17A in this same group presented a significant reduction, which may indicate that the problem is not the overproduction of proinflammatory cytokines but an alteration in the regulation of this cytokine profile.

CONCLUSION

Periodontitis could be associated with clinical signs of placental infection and APO. Periodontal infection by *P. gingivalis* can induce atopobiosis (translocation) to the placenta and trigger inflammation, although a direct relationship with the occurrence of APO cannot be proven.

There is a pro-inflammatory pattern associated with labor, characterized by Th-1 profile and the activity of cytotoxic cells, which is enhanced by placental infection with UGM. However, PI associated with *P. gingivalis* suggests a switch where the balance of Th-1 profile favors an inflammatory response mediated by MCP-1 and macrophage activity as a mechanistic explanation of its possible relationship with adverse outcomes during pregnancy.

Strengths and Limitations

One of the major limitations of this study was the size of the groups. Despite the number of patients included in the initial phase, the prevalence of PI was low and low concentrations of bacterial DNA were obtained. Although 12 species of microorganisms associated with PI were evaluated in this study, more could be accomplished using nested PCR-species-specific, sequencing and metagenomic techniques which could provide more information on the placental microbiome; the use of these techniques is suggested for future studies that evaluate

immune response in the placenta in the presence of infection. Although the size of the groups was small, the results of this study are the first to be published on a differential pattern associated with human placental infection by *P. gingivalis* associated with APO or CA.

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 Ethics Committee of Universidad El Bosque (PCI-2014-18). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The authors certify that they have participated sufficiently in the work to take public responsibility for the appropriateness of the design and method of review, as well as, the reviewing of the manuscript and approve it for publication. 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/fmicb.2020.591626/full#supplementary-material

REFERENCES

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., and Versalovic, J. (2014). The placenta harbors a unique microbiome. *Sci. Transl. Med.* 6:237ra65. doi: 10.1126/scitranslmed.3008599

Allen-Daniels, M. J., Serrano, M. G., Pflugner, L. P., Fettweis, J. M., Prestosa, M. A., Koparde, V. N., et al. (2015). Identification of a gene in mycoplasma hominis associated with preterm birth and microbial burden in intraamniotic infection. *Am. J. Obstet. Gynecol.* 212, 779.e1–779.e13. doi: 10.1016/j.ajog.2015.01.032

Ao, M., Miyauchi, M., Furusho, H., Inubushi, T., Kitagawa, M., Nagasaki, A., et al. (2015). Dental infection of *Porphyromonas gingivalis* induces preterm birth in mice. *PLoS One* 10:e0137249. doi: 10.1371/journal.pone.0137249

Arce, M., Caron, K., Barros, S., and Offenbacher, S. (2012). TLR4 mediate intrauterine growth restriction after systemic *C. rectus* infection in mice. *Mol. Oral Microbiol.* 27, 373–381. doi: 10.1111/j.2041-1014.2012.00651.x

Arenas-Hernandez, M., Romero, R., St Louis, D., Hassan, S., Kaye, E., and Gomez-Lopez, N. (2015). An imbalance between innate and adaptive immune cells at the maternal-fetal interface occurs prior to endotoxin-induced preterm birth. *Cell. Mol. Immunol.* 13, 462–473. doi: 10.1038/cmi.2015.22

Ashimoto, A., Chen, C., Bakker, I., and Slots, J. (1996). Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol. Immunol.* 11, 266–273. doi: 10.1111/j.1399-302X.1996.tb00180.x

Audigier, V., Husson, F., and Josse, J. (2016). A principal component method to impute missing values for mixed data. *Adv. Data Anal Classif.* 10, 5–26. doi: 10.1007/s11634-014-0195-1

Azizieh, F. Y., and Raghupathy, R. (2017). IL-10 and pregnancy complications. *Clin. Exp. Obstet. Gynecol.* 44, 252–258. doi: 10.12891/ceog3456.2017

Balan, P., Chong, Y. S., Umashankar, S., Swarup, S., Loke, W. M., Lopez, V., et al. (2018). Keystone species in pregnancy gingivitis: a snapshot of oral microbiome during pregnancy and postpartum period. *Front. Microbiol.* 9:2360. doi: 10.3389/fmicb.2018.02360

Bardos, J., Fiorentino, D., Longman, R. E., and Paidas, M. (2020). Immunological role of the maternal uterine microbiome in pregnancy: pregnancies pathologies and alterated microbiota. *Front. Immunol.* 10:2823. doi: 10.3389/fimmu.2019.02823

Bardou, M., Hadi, T., Mace, G., Pesant, M., Debermont, J., Barrichon, M., et al. (2014). Systemic increase in human maternal circulating CD14+CD16-MCP-1+ monocytes as a marker of labor. *Am. J. Obstet. Gynecol.* 210, 70.e1–70.e9. doi: 10.1016/j.ajog.2013.08.031

Blanchard, A., Yáñez, A., Dybvig, K., Watson, H. L., Griffiths, G., and Cassell, G. H. (1993). Evaluation of intraspecies genetic variation within the 16S rRNA gene of mycoplasma hominis and detection by polymerase chain reaction. *J. Clin. Microbiol.* 31, 1358–1361. doi: 10.1128/JCM.31.5.1358-1361.1993

Cao, B., Stout, M., Lee, I., and Mysorekar, I. (2014). Placental microbiome and its role in preterm birth. *Neo Rev.* 15, e537–e545. doi: 10.1542/neo.15-12-e537

Cappelletti, M., Della Bella, S., Ferrazzi, E., Mavilio, D., and Divanovic, S. (2016). Inflammation and preterm birth. *J. Leukoc. Biol.* 99, 67–78. doi: 10.1189/jlb.3MR0615-272RR

Chopra, A., Radhakrishnan, R., and Sharma, M. (2020). *Porphyromonas gingivalis* and adverse pregnancy outcomes: a review on its intricate pathogenic mechanisms. *Crit. Rev. Microbiol.* 46, 213–236. doi: 10.1080/1040841X.2020.1747392

Combs, C. A., Gravett, M., Garite, T. J., Hickok, D. E., Lapidus, J., Porreco, R., et al. (2014). Amniotic fluid infection, inflammation, and colonization in preterm labor with intact membranes. *Am. J. Obstet. Gynecol.* 210, 125.e1–125.e15. doi: 10.1016/j.ajog.2013.11.032

DiGiulio, D. B., Romero, R., Amogan, H. P., Kusanovic, J. P., Bik, E. M., Gotsch, F., et al. (2008). Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS One* 3:e3056. doi: 10.1371/journal.pone.0003056

Doyle, R. M., Alber, D. G., Jones, H. E., Harris, K., Fitzgerald, F., Peebles, D., et al. (2014). Term and preterm labour are associated with distinct microbial community structures in placental membranes which are independent of mode of delivery. *Placenta* 35, 1099–1101. doi: 10.1016/j.placenta.2014.10.007

Eke, P. I., Page, R. C., Wei, L., Thornton-Evans, G., and Genco, R. J., (2010). Update of the case definitions for population-based surveillance of periodontitis. *J. Periodontol.* 83, 1449–1454. doi: 10.1902/jop.2012.110664

Erlebacher, A. (2013). Immunology of the maternal-fetal interface. *Annu. Rev. Immunol.* 31, 387–411. doi: 10.1146/annurev-immunol-032712-100003

Esplin, M., Romero, R., Chaiworapongsa, T., Kim, Y., Edwin, S., Gomez, R., et al. (2005). Monocyte chemotactic protein-1 is increased in the amniotic

fluid of women who deliver preterm in the presence or absence of intra-amniotic infection. *J. Matern. Fetal Neonatal Med.* 17, 365–373. doi: 10.1080/14767050500141329

Farr, A., Kiss, H., Holzer, I., Husslein, P., Hagmann, M., and Petricevic, L. (2015). Effect of asymptomatic vaginal colonization with *Candida albicans* on pregnancy outcome. *Acta Obstet. Gynecol. Scand.* 94, 989–996. doi: 10.1111/aogs.12697

Fischer, L. A., Demerath, E., Bittner-Eddy, P., and Costalonga, M. (2019). Placental colonization with periodontal pathogens: the potential missing link. *Am. J. Obstet. Gynecol.* 221, 383.e3–392.e3. doi: 10.1016/j.ajog.2019.04.029

Fredricks, D. N., Fiedler, T. L., Thomas, K. K., Oakley, B. B., and Marrazzo, J. M. (2007). Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *J. Clin. Microbiol.* 45, 3270–3276. doi: 10.1128/JCM.01272-07

Fukui, A., Ntrivalas, E., Fukuhara, R., Fujii, S., Mizunuma, H., Gilman-Sachs, A., et al. (2009). Correlation between natural cytotoxicity receptors and intracellular cytokine expression of peripheral blood NK cells in women with recurrent pregnancy losses and implantation failures. *Am. J. Reprod. Immunol.* 62, 371–380. doi: 10.1111/j.1600-0897.2009.00750.x

Gaudilliere, D. K., Culos, A., Djebali, K., Tsai, A. S., Ganio, E. A., Choi, W. M., et al. (2019). Systemic immunologic consequences of chronic periodontitis. *J. Dent. Res.* 98, 985–993. doi: 10.1177/0022034519857714

Gibbs, R. S., and Duff, P. (1991). Progress in pathogenesis and management of clinical intraamniotic infection. *Am. J. Obstet. Gynecol.* 164, 1317–1326. doi: 10.1016/0002-9378(91)90707-X

Gomez-Arango, L. F., Barrett, H. L., McIntyre, H. D., Callaway, L. K., Morrison, M., and Nitert, M. D. (2017). Contributions of the maternal oral and gut microbiome to placental microbial colonization in overweight and obese pregnant women. *Sci. Rep.* 7:2860. doi: 10.1038/s41598-017-03066-4

Gomez-Lopez, N., StLouis, D., Lehr, M. A., Sanchez-Rodriguez, E. N., and Arenas-Hernandez, M. (2014). Immune cells in term and preterm labor. *Cell. Mol. Immunol.* 11, 571–581. doi: 10.1038/cmi.2014.46

Hajishengallis, G. (2014). Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. *Trends Immunol.* 35, 3–11. doi: 10.1016/j.it.2013.09.001

Hajishengallis, G. (2015). Periodontitis: from microbial immune subversion to systemic inflammation. *Nat. Rev. Immunol.* 15, 30–44. doi: 10.1038/nri3785

Hajishengallis, G., Darveau, R. P., and Curtis, M. A. (2012). The keystone-pathogen hypothesis. *Nat. Rev. Microbiol.* 10, 717–725. doi: 10.1038/nrmicro2873

Hanna, N., Bonifacio, L., Weinberger, B., Reddy, P., Murphy, S., Romero, R., et al. (2006). Evidence for interleukin-10-mediated inhibition of cyclo-oxygenase-2 expression and prostaglandin production in preterm human placenta. *Am. J. Reprod. Immunol.* 55, 19–27. doi: 10.1111/j.1600-0897.2005.00342.x

Hasegawa-Nakamura, K., Tateishi, F., Nakamura, T., Nakajima, Y., Kawamata, K., Douchi, T., et al. (2011). The possible mechanism of preterm birth associated with periodontopathic *Porphyromonas gingivalis*. *J. Periodontal Res.* 46, 497–504. doi: 10.1111/j.1600-0765.2011.01366.x

Helmo, F. R., Alves, E. A. R., Moreira, R. A. A., Severino, V. O., Rocha, L. P., and Monteiro, M. L. G. D. R. (2018). Intrauterine infection, immune system and premature birth. *J. Matern. Fetal Neonatal Med.* 31, 1227–1233. doi: 10.1080/14767058.2017.1311318

Horliana, A. C., Chambrone, L., Foz, A. M., Artese, H. P., Rabelo, M. S., Pannuti, C. M., et al. (2014). Dissemination of periodontal pathogens in the bloodstream after periodontal procedures: a systematic review. *PLoS One* 28:e98271. doi: 10.1371/journal.pone.0098271

Jacobsson, B., Holst, R., Wennerholm, U., Andersson, B., Lilja, H., and Hagberg, H. (2003). Monocyte chemotactic protein-1 in cervical and amniotic fluid: relationship to microbial invasion of the amniotic cavity, intra-amniotic inflammation, and preterm delivery. *Am. J. Obstet. Gynecol.* 189, 1161–1167. doi: 10.1067/S0002-9378(03)00594-5

Katz, J., Chegini, N., Shiverick, K., and Lamont, R. (2009). Localization of *P. gingivalis* in preterm delivery placenta. *J. Dent. Res.* 88, 575–578. doi: 10.1177/0022034509338032

Kuriyama, T., Karasawa, T., Nakagawa, K., Kawashiri, S., Nakanishi, I., Nakamura, S., et al. (2000). Characterization of bacterial orofacial infections using a new murine model. *Microb. Pathog.* 29, 115–120. doi: 10.1006/mpat.2000.0375

Lafaurie, G. I., Contreras, A., Barón, A., Botero, J., Mayorga-Fayad, I., Jaramillo, A., et al. (2007). Demographic, clinical, and microbial aspects of chronic and aggressive periodontitis in Colombia: a multicenter study. *J. Periodontol.* 78, 629–639. doi: 10.1902/jop.2007.060187

Lannon, S. M., Vanderhoeven, J. P., Eschenbach, D. A., Gravett, M. G., and Adams Waldorf, K. M. (2014). Synergy and interactions among biological pathways leading to preterm premature rupture of membranes. *Reprod. Sci.* 21, 1215–1227. doi: 10.1177/1933719114534535

Leiby, J. S., McCormick, K., Sherrill-Mix, S., Clarke, E. L., Kessler, L. R., Taylor, L. J., et al. (2018). Lack of detection of a human placenta microbiome in samples from preterm and term deliveries. *Microbiome* 6:196. doi: 10.1186/s40168-018-0575-4

Leon, L. J., Doyle, R., Diez-Benavente, E., Clark, T. G., Klein, N., Stanier, P., et al. (2018). Enrichment of clinically relevant organisms in spontaneous preterm-delivered placentas and reagent contamination across all clinical groups in a large pregnancy cohort in the United Kingdom. *Appl. Environ. Microbiol.* 84, e00483–e00518. doi: 10.1128/AEM.00483-18

León, R., Silva, N., Ovalle, A., Chaparro, A., Ahumada, A., Gajardo, M., et al. (2007). Detection of *Porphyromonas gingivalis* in the amniotic fluid in pregnant women with a diagnosis of threatened premature labor. *J. Periodontol.* 78, 1249–1255. doi: 10.1902/jop.2007.060368

Li, S., Wang, W., Fu, S., Wang, J., Liu, H., Xie, S., et al. (2013). IL-21 modulates release of proinflammatory cytokines in lps-stimulated macrophages through distinct signaling pathways. *Mediat. Inflamm.* 2013:548073. doi: 10.1155/2013/548073

Liang, S., Ren, H., Guo, H., Xing, W., Liu, C., Ji, Y., et al. (2018). Periodontal infection with *Porphyromonas gingivalis* induces preterm birth and lower birth weight in rats. *Mol. Oral Microbiol.* 33, 312–321. doi: 10.1111/omi.12227

Liu, Y., Liu, Y., Zhang, R., Zhu, L., and Feng, Z. (2017a). Early- or mid-trimester amniocentesis biomarkers for predicting preterm delivery: a meta-analysis. *Ann Med.* 49, 1–10. doi: 10.1080/07853890.2016.1211789

Liu, Z. Z., Sun, G. Q., Hu, X. H., Kwak-Kim, J., and Liao, A. H. (2017b). The transdifferentiation of regulatory T and Th-17 cells in autoimmune/inflammatory diseases and its potential implications in pregnancy complications. *Am. J. Reprod. Immunol.* 78:2. doi: 10.1111/aji.12657

Mahmoundi-Rad, M., Zafarghandi, A. S., Amel Zabihi, M., Tavallaee, M., and Mirdamadi, Y. (2012). Identification of Candida species associated with vulvovaginal candidiasis by multiplex PCR. *Infect. Dis. Obstet. Gynecol.* 2012:872169. doi: 10.1155/2012/872169

McCormick, M. C. (1985). The contribution of low birth weight to ant mortality and childhood morbidity. *N. Engl. J. Med.* 312, 82–90. doi: 10.1056/NEJM198501103120204

Mendelson, C. R. (2009). Minireview: fetal-maternal hormonal signaling in pregnancy and labor. *Mol. Endocrinol.* 23, 947–954. doi: 10.1210/me.2009-0016

Montenegro, D. A., Borda, L. F., Neuta, Y., Gómez, L. A., Castillo, D. M., Loyo, D., et al. (2019). Oral and uro-vaginal intra-amniotic infection in women with preterm delivery: a case-control study. *J. Investig. Clin. Dent.* 10:e12396. doi: 10.1111/jicd.12396

Morgan, T. K. (2016). Role of the placenta in preterm birth: a review. *Am. J. Perinatol.* 33, 258–266. doi: 10.1055/s-0035-1570379

Park, H., Park, K. H., Kim, Y. M., Kook, S. Y., Jeon, S. J., and Yoo, H. N. (2018). Plasma inflammatory and immune proteins as predictors of intra-amniotic infection and spontaneous preterm delivery in women with preterm labor: a retrospective study. *BMC Pregnancy Childbirth* 18:146. doi: 10.1186/s12884-018-1780-7

Parnell, L. A., Briggs, C. M., Cao, B., Delannoy-Bruno, O., Schrieffer, A. E., and Mysorekar, I. U. (2017). Microbial communities in placentas from term normal pregnancy exhibit spatially variable profiles. *Sci. Rep.* 7:11200. doi: 10.1038/s41598-017-11514-4

Parris, K. M., Amabebe, E., Cohen, M. C., and Anumba, D. O. (2020). Placental microbial-metabolite profiles and inflammatory mechanisms associated with preterm birth. *J. Clin. Pathol.* doi: 10.1136/jclinpath-2020-206536 [Epub ahead of print]

Piccinni, M. P., Maggi, E., and Romagnani, S. (2000). Role of hormone-controlled T-cell cytokines in the maintenance of pregnancy. *Biochem. Soc. Trans.* 28, 212–215. doi: 10.1042/bst0280212

Potgieter, M., Bester, J., Kell, D. B., and Pretorius, E. (2015). The dormant blood microbiome in chronic, inflammatory diseases. *FEMS Microbiol. Rev.* 39, 567–591. doi: 10.1093/femsre/fuv013

Prince, A. L., Ma, J., Kannan, P. S., Alvarez, M., Gisslen, T., Harris, R. A., et al. (2016). The placental membrane microbiome is altered among subjects with spontaneous preterm birth with without chorioamnionitis. *Am. J. Obstet. Gynecol.* 214, 627.e1–627.e16. doi: 10.1016/j.ajog.2016.01.193

Ragsdale, H. B., Kuzawa, C. W., Borja, J. B., Avila, J. L., and McDade, T. W. (2019). Regulation of inflammation during gestation and birth outcomes:

inflammatory cytokine balance predicts birth weight and length. *Am. J. Hum. Biol.* 31:e23245. doi: 10.1002/ajhb.23245

Saito, S., Nakashima, A., Shima, T., and Ito, M. (2010). Th-1/Th2/Th-17 and regulatory T-cell paradigm in pregnancy. *Am. J. Reprod. Immunol.* 63, 601–610. doi: 10.1111/j.1600-0897.2010.00852.x

Seferovic, M. D., Pace, R. M., Carroll, M., Belfort, B., Major, A. M., Chu, D. M., et al. (2019). Visualization of microbes by 16S in situ hybridization in term and preterm placentas without intraamniotic infection. *Am. J. Obstet. Gynecol.* 221, 146.e1–146.e23. doi: 10.1016/j.ajog.2019.04.036

Stinson, L. F., Boyce, M. C., Payne, M. S., and Keelan, J. A. (2019). The not-so-sterile womb: evidence that the human fetus is exposed to bacteria prior to birth. *Front. Microbiol.* 10:1124. doi: 10.3389/fmicb.2019.01124

Stout, M. J., Conlon, B., Landeau, M., Lee, I., Bower, C., Zhao, Q., et al. (2013). Identification of intracellular bacteria in the basal plate of the human placenta in term and preterm gestations. *Am. J. Obstet. Gynecol.* 208, 226.E1–226.E7. doi: 10.1016/j.ajog.2013.01.018

Teng, L. J., Zheng, X., Glass, J. I., Watson, H. L., Tsai, J., and Cassell, G. H. (1994). Urea plasma urealyticum biovar specificity and diversity are encoded in multiple banded antigen gene. *J. Clin. Microbiol.* 1994, 1464–1469. doi: 10.1128/JCM.32.6.1464-1469.1994

Theis, K. R., Romero, R., Winters, A. D., Greenberg, J. M., Gomez-Lopez, N., Alhousseini, A., et al. (2019). Does the human placenta delivered at term have a microbiota? Results of cultivation, quantitative real-time PCR, 16S rRNA gene sequencing, and metagenomics. *Am. J. Obstet. Gynecol.* 220, 267.e1–267.e39. doi: 10.1016/j.ajog.2018.10.018

Vanterpool, S. F., Been, J. V., Houben, M. L., Nikkels, P. G., De Krijger, R. R., Zimmermann, L. J., et al. (2016). Porphyromonas gingivalis within placental villous mesenchyme and umbilical cord stroma is associated with adverse pregnancy outcome. *PLoS One* 11:e0146157. doi: 10.1371/journal.pone.0146157

Walsh, S. W., Nugent, W. H., Alam, S. M. K., Washington, S. L., Teves, M., Jefferson, K. K., et al. (2020). Protease amplification of the inflammatory response induced by commensal bacteria: implications for racial disparity in term and preterm birth. *Reprod. Sci.* 27, 246–259. doi: 10.1007/s43032-019-00011-w

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REVIEW
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Oral Dysbiosis and Autoimmunity: From Local Periodontal Responses to an Imbalanced Systemic Immunity. A Review

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The current paradigm of onset and progression of periodontitis includes oral dysbiosis directed by inflammophilic bacteria, leading to altered resolution of inflammation and lack of regulation of the inflammatory responses. In the construction of explanatory models of the etiopathogenesis of periodontal disease, autoimmune mechanisms were among the first to be explored and historically, for more than five decades, they have been described in an isolated manner as part of the tissue damage process observed in periodontitis, however direct participation of these mechanisms in the tissue damage is still controversial. Autoimmunity is affected by genetic and environmental factors, leading to an imbalance between the effector and regulatory responses, mostly associated with failed resolution mechanisms. However, dysbiosis/infection and chronic inflammation could trigger autoimmunity by several mechanisms including bystander activation, dysregulation of toll-like receptors, amplification of autoimmunity by cytokines, epitope spreading, autoantigens complementarity, autoantigens overproduction, microbial translocation, molecular mimicry, superantigens, and activation or inhibition of receptors related to autoimmunity by microorganisms. Even though autoreactivity in periodontitis is biologically plausible, the associated mechanisms could be related to non-pathologic responses which could even explain non-recognized physiological functions. In this review we shall discuss from a descriptive point of view, the autoimmune mechanisms related to periodontitis physio-pathogenesis and the participation of oral dysbiosis on local periodontal autoimmune responses as well as on different systemic inflammatory diseases.

Keywords: autoimmunity, autoantigens, autoantibodies, oral, dysbiosis, microbiome, periodontitis, *Porphyromonas gingivalis*

INTRODUCTION

The immune system has an uneasy relationship with the environment (1), being a mobile network of cells with emergent properties derived from dynamic cellular interactions that have evolved to guard the body against attack (2). Under normal conditions, the immune system exhibits tolerance, and must be able to distinguish self from non-self and harmless non-self from dangerous non-self (1). When self-tolerance is disrupted, autoimmunity will arise (3). The alteration in the activation of almost any immune process in a susceptible host could trigger immune dysregulation and autoimmunity. This fact is more clearly understood from the analysis of primary immunodeficiency disorders, initially described in patients who had severe or recurrent infections from which the contradiction arises that individuals who were unable to respond against foreign antigens, did so against their own antigens. Immunodeficiencies in which the flaws are not in the effector response but in the immune regulatory mechanisms, have been called immune dysregulation disorders (4, 5). Autoimmunity and immunodeficiencies are today considered two sides of the same coin, being conditions that are not mutually exclusive and that are both linked to alterations in signaling mechanisms and immune regulation; genetic defects have been described not only as the cause of primary immunodeficiency syndromes, but also as factors that predispose to infections and lead to dysregulation and autoimmunity (6).

The host-microbiome relationship must be understood today far beyond the concept of disease. The microbiome is related to multiple functions in the host, including the control of vascularization at the intestinal level (7) and the functioning of the central nervous system affecting the production of soluble mediators (8); but without a doubt, the most recognized role of the commensal microbiome in maintaining healthy homeostasis is its immunostimulatory effect. This includes the recruitment of mucosal immune cells, the generation and maturation of organized lymphoid tissues (9), and the stimulation of protective functions of epithelial cells such as mucus formation and production of antimicrobial peptides (10).

Given the recognition of the role of commensal microbiota in the development of immunity and the regulation of the immune response against pathogens, it is not unreasonable to think that this microbiota is also capable of regulating/controlling autoimmune responses. The participation of microorganisms in the occurrence of autoimmunity is being widely supported, as key actors in the loss of microbiome-host homeostasis.

Periodontal disease was recognized for decades as an infectious disease, but more than half a century ago, relationships began to be established with the host's response to infecting microorganisms, as a determining part of its pathogenesis. In 1965, Brandtzaeg and Kraus (11) made an approach to the immunological mechanisms in periodontal disease, describing for the first time the autoimmune basis in the pathogenesis of periodontitis. They described the presence of anti-collagen antibodies produced by plasma cells in the periodontal tissue of patients with periodontitis. From this, the possible development of an autoimmune process was considered, based on the destructive consequences of this disease in the alveolar bone, conceived at that time as a localized hypersensitivity reaction that involved the formation of immune complexes in the affected periodontal tissues (12).

As described not even a decade ago, today we understand periodontitis as the result of dysbiosis of the oral microbiota guided by inflammophilic bacteria, leading to an altered resolution of inflammation and lack of regulation of the inflammatory responses (13). The damage is directly related to the activation of inflammatory mechanisms by the host, however not fully elucidated (e.g. the role of autoimmunity in periodontitis pathogenesis) (**Figure 1**).

This review aims to summarize the host and oral microbiome changes involved in the physio-pathogenesis of periodontitis that have been described as contributing factors in local and systemic auto-immune responses leading to disease, as well as to analyze them beyond tissue damage from the physiological activation of the immune system.

Microbiome-Host Relationship in Autoimmune Responses

Autoimmune processes are the result of a failure in the immune mechanisms of self-tolerance towards components of different nature (proteins, receptors, tissue, etc.) where the microbiota has frequently been involved. The mechanisms by which microorganisms participate in the control of autoimmunity are still unclear, but several approaches have been described. One of the most important, starts from the development of tolerance to infection (14), where resident microorganisms play a very important role by hindering, and even avoiding colonization of their habitat by exercising colonization resistance (15).

It has been observed in animals and humans with genetic predisposition, that the microbiota provides signals that induce antimicrobial effectors that are neutralized by inhibitory microbial signals, establishing as a result, a homeostatic relationship with the host (14). If a specific microorganism's line expands, this blocks the development of autoimmunity and improves their own chances of staying in that expanded state suppressing the host's adaptive and inflammatory responses (16). This hypothesis is known as the "specific lineage hypothesis", in which autoimmunity is "turned off" as a side effect. It could also happen that there is a "signal balance" where, while the host response is balanced against commensals, their effort to reduce it

Abbreviations: aCL, Anti-cardiolipin antibodies; ACPA, Anti-citrullinated protein antibodies; APCs, Antigen presenting cells; ANCAs, Anti-neutrophil cytoplasmatic antibodies; oxLDL, Anti-oxidized LDL; CTL4, cytotoxic T lymphocyte – associated protein 4; DNA, Deoxyribonucleic acid; GAD65, Enzyme glutamate decarboxylase; HSP, Heat shock proteins; IgG, Immunoglobulin G; IR, Inhibitory receptors; IFN-γ, Interferon gamma; IL, Interleukin; LPS, Lipopolysaccharides; MMPs, Matrix metalloproteinases; MS, Multiple sclerosis; MDSCs, Myeloid derived suppressor cells; NETs, Neutrophil extracellular traps; ox-LDL, Oxidized low-density lipoprotein; PRR, Pattern recognition receptor; PD-1, programmed cell death protein 1; PADs, Peptidyl-arginine deiminase; PR3, Proteinase 3; REGA, Remnant epitopes generate autoimmunity; RA, Rheumatoid arthritis; SLE, Systemic lupus erythematosus; TCR, T cell receptor; Th17, T helper 17 cells; TGF-β, Transforming growth factor beta; TNF-α, Tumor necrosis factor-α; V, Variable region.

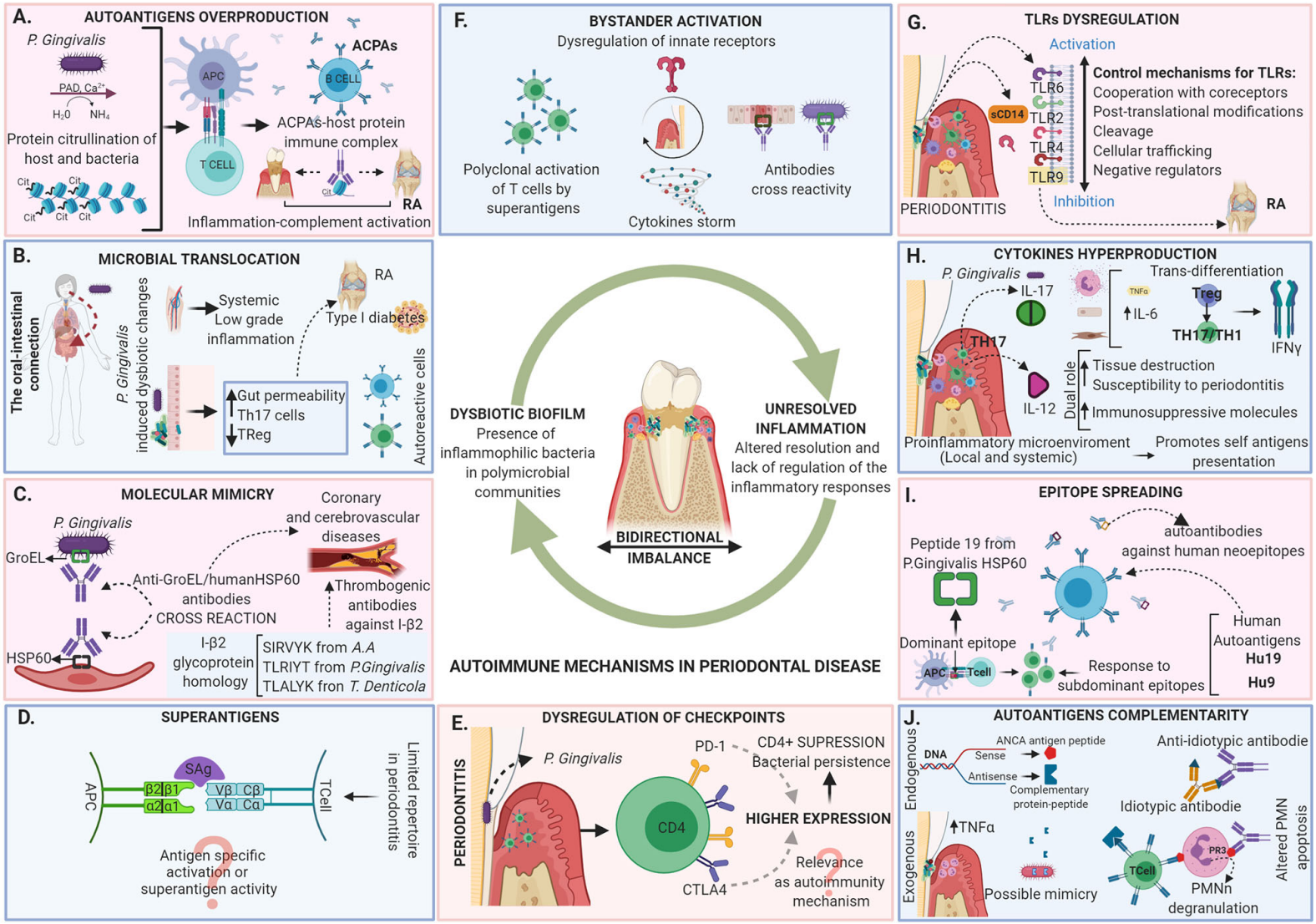

FIGURE 1 | Autoimmune mechanisms in periodontal disease. Periodontitis is today described as the result of dysbiosis of the oral microbiota guided by inflammophilic bacteria, leading to an altered resolution of inflammation and a lack of regulation of the inflammatory response. The tissue damage in periodontitis is directly related to the activation of inflammatory mechanisms by the host that are in a bidirectional imbalance with the microbiome. The dysregulation leads to the appearance of local and systemic autoimmune responses mediated by different mechanisms including **(A)** Autoantigens overproduction, **(B)** Microbial translocation, **(C)** Molecular mimicry, **(D)** Superantigens, **(E)** Dysregulation of checkpoints, **(F)** Bystander activation, **(G)** TLRs dysregulation, **(H)** Cytokines hyperproduction, **(I)** Epitope spreading and **(J)** Autoantigens complementarity. The evidence of the activation of the diverse autoimmune mechanisms in the periodontal tissues does not necessarily indicate that they are related to local tissue damage, although they may be related to the development of autoimmune responses at the systemic level associated with the occurrence of multiple diseases.

does not affect the development of the disease but the host's inability to control the microbiota correctly, resulting in the dominance of negative signals provided by microorganisms and the concomitant reduction of autoimmunity (17).

Although the microbiome is strongly involved in the maintenance of health and apparently in the control of autoimmunity, on the other hand, there is sufficient evidence in both humans (18, 19) and animals (20, 21) that supports the role of microorganisms in the occurrence of autoimmune responses. An expansion of pathobionts has recently been found in patients with autoimmune diseases and in animal models of autoimmunity (22).

Microbiome and Oral Dysbiosis

The human microbiome is made up of all the microorganisms that live in the human body occupying multiple systems. As the name implies, they are communities -biomes- of microorganisms with very complex interactions with each other and with the host (23). The composition of the human microbiome is highly influenced by changes in the microenvironment. The microflora can be transitory, permanent, pathogenic or non-pathogenic, which has been evolvingly adapted to humans, these being the true commensal microorganisms. Of the latter, some have the ability to modulate the immune response without producing changes in homeostasis known as autobionts, while others are transient pathogens or pathobionts (24) which are not normally pathogenic *per se*, but can trigger immune-inflammatory processes, including autoimmunity in genetically susceptible individuals (22).

The change in the characteristic microorganism communities of a particular microenvironment is known as dysbiosis, also called dysbacteriosis. Bacterial translocation, related to the pathogenesis of multiple diseases, could be better described as atopobiosis, which is the appearance of microorganisms that are characteristic of a certain microenvironment in the "wrong" place. Atopobiosis can occur by multiple ways (25, 26), and is perhaps one of the most relevant mechanisms in the participation of the oral microbiota in multiple conditions and diseases.

It has been demonstrated that microorganisms that are in low numbers in the metagenome can be critical in the community for carrying out essential metabolic activities (18). The mouth of a healthy individual may be colonized by about 100-200 species of the more than 700 oral bacteria identified (27), with inter-individual variations resulting from the environment, genetics, age, and lifestyles (28, 29). The presence of certain mechanisms could prevent dysbiotic changes in the community even in the presence of disease triggers (e.g. greater production of nitric oxide) (30) and their interrelation with the host is then a determining factor in the health-disease process. In fact, homeostasis is triggered by health maintenance mechanisms contributing to resilience especially in tolerant individuals. These mechanisms may be dependent on the microorganisms but also on the host marked by genetic and epigenetic differences, and their identification could lead to the development of new therapeutic strategies aimed at promoting health rather than reducing disease (31).

In the oral cavity and other systems exposed to the external environment, the immune system is constantly exposed to signals that go beyond the commensal-epithelial interaction, including constant damage to tissues resulting from chewing, and antigens from both food and airborne particles. This phenomenon participates in the training of the system by mechanisms that are not entirely clear (32); but what is clear is that the breakdown of the balance of these local responses is related to susceptibility to certain tissue-specific diseases, periodontitis being the prototype, and whose pathogenesis reflects the activation of local and systemic inflammatory processes (including manifestations of autoimmunity), indicating the occurrence of dysregulation of the immune system at this level (33).

Thus, the conception of periodontal disease has changed, relating it now less to a specific bacteria, but more to heterotypic (polymicrobial) groups of microorganisms resulting in dysbiotic communities that alter tissue homeostasis and normal immune responses (34, 35). It has been described that there are community members, specifically *P. gingivalis* (a suggested keystone pathogen in animal models), that even in low numbers (<1%), can increase the virulence of the entire community (36); communication between it and normally commensal microorganisms (accessory pathogens) facilitate synergy and the transition to pathogenicity, allowing the continued development of the dysbiotic community and stimulating the inflammatory response, in which misdirected responses that contribute to tissue destruction are mixed and shape a modified microbiota with "inflammophilic" characteristics, that uses nutrients derived from tissue damage for its survival. The virulence of the community rises as a consequence of the subversion of specific components of the immune response, and thus, this dysbiotic community increases causing additional damage and greater alteration to the homeostasis of the system, mediated mainly by pathobionts (*Filifactor alocis, Peptostreptococcus stomatis*, species of the genus *Prevotella, Megasphaera, Selenomonas*, and *Desulfobulbus*), usually underestimated in periodontitis, but with an apparent better correlation with the disease (37, 38).

The virulence of *P. gingivalis* becomes important when a synergistic microbial community is formed in a susceptible host, indicating that both are necessary for the expression of the microorganism pathogenicity (39). However, other species might be equally or even more active in the process that leads from periodontal health to disease and should be investigated. Besides, it should be noted that the "keystone pathogenesis" itself has yet to be demonstrated in humans (34).

The dysbiosis is closely related to periodontitis, and the relationship between the subgingival microbiome and the immune and inflammatory response seems to be important in the pathogenesis of the disease. The evidence has allowed to hypothesize that microbiome and inflammation in periodontal health are in a bidirectional balance and in periodontitis in a bidirectional imbalance, which seems to be common in other diseases such as sepsis, inflammatory bowel disease, etc (22) (**Figure 1**).

The autoimmunity mechanisms linked to dysbiosis are being actively studied, always having as a basis conditions where the end point is the destruction of the cells of the target tissues by self-reactive cells or autoantibodies, and that involve the participation of microorganisms' dependent mechanisms of the immune system, both innate and adaptive. Understanding that the microbiome-host interrelation is responsible for the occurrence of tissue damage in dysbiosis related infectious diseases, we grouped the autoimmunity mechanisms into those led by the microorganisms and those where the baton is carried by the immune system, and from the evidence will be related with the autoimmune responses observed in tissue damage/regulation in periodontitis and periodontitis linked systemic diseases.

Microbial-Dependent Mechanisms of Autoimmunity

Autoantigens Overproduction

The overproduction of autoantigens may well occur because the microorganisms produce enzymes that can break the extracellular matrix (i.e. fibrinogen, fibronectin, and type I collagen), creating "remnant epitopes" that act as autoantigens or because of an enzymatic modification of antigens (e.g. citrullination) that ends in the generation of autoantibodies (40). In health, the matrix metalloproteinases (MMPs) produced by the host have a role in tissue development, homeostasis and remodeling, cell migration and tissue healing, activation of immune cells and defense against pathogens, in a highly regulated form. When regulation is lost, high levels of these proteases lead to cellular destruction described in diseases of various kinds, from periodontitis to cancer (41), and why not autoimmunity (**Figure 2A**).

Microorganisms also produce MMPs causing direct tissue damage (close to 1%), but more importantly they activate circulating immune cells to produce MMPs which are responsible for the greatest amount of tissue rupture (42); then, chronic inflammatory processes lead to the production of cytokines and chemokines secreted by phagocytes, that recruit and stimulate leukocytes to release proteinases that act together with the MMPs (43). These events result in the proteolysis of matrix molecules and degradation of intact proteins into remnant fragments (2). These Remnant Epitopes Generated Autoimmunity (REGA model) explain the generation of autoantigens and their interaction with the T-cell receptor complex. In this model, cytokine and chemokine regulated proteases play a central role in the generation of autoantigens (44). Initially, evidence was documented for this model in multiple sclerosis (MS), however, it has been applied to other autoimmune diseases like rheumatoid arthritis (RA) and type I diabetes (45).

Recently the model was revised and reaffirms that it is an example of how remnant epitopes generate, maintain and regulate autoimmunity, in which the cells of innate immunity and not lymphocytes or their molecules initiate autoimmune reactions by cytokine-mediated proteolysis which consequently leaves remnant epitopes (46). Although REGA is not fully described in periodontitis, the high degree of destruction observed in periodontal tissues mediated by different proteinases makes it quite plausible.

On the other hand, the post-translational modification of proteins by the enzymatic deamination of arginine residues converts positively charged peptidyl arginine to neutral peptidyl citrulline (47). This process is called citrullination and is catalyzed by the enzyme peptidylarginine deiminase (PAD) (48). Various protein candidates for citrullination, such as keratin, fibrinogen, vimentin, fibronectin, and α-enolase have been identified (49).

Apart from being involved in many physiological processes (skin keratinization, brain development and in gene regulation *via* chromatin remodeling), citrullination can occur under pathologic inflammatory conditions associated with apoptosis, necrosis of neutrophils and NETosis (50). During the latter process, hypercitrullination of proteins is needed for the formation of neutrophil extracellular traps (NETs) (47) which are extracellular fibers generated by activated polymorphonuclear neutrophils, composed of nuclear constituents that extracellularly immobilize, disarm and kill microbial pathogens (48), meaning that under normal conditions NETs have antimicrobial function. However, uncontrolled NETs formation might contribute to tissue damage and provide a source of autoantigens; the citrullination of proteins and peptides has been linked to RA, primary open-angle glaucoma, nephropathy, MS, Alzheimer's disease, and psoriasis (47).

The breakdown of immune tolerance in genetically susceptible individuals initiates the generation of anti-citrullinated protein antibodies in the synovia and contributes to the subsequent development of RA (49). These autoantibodies are known as ACPA and occur in ~ 70% of patients with RA. ACPA are known to be a sensitive and specific marker that can be detected in the circulation years before the clinical onset of the disease (47). During the clinical course of RA they strongly correlate with disease severity (50).

Five different PAD have been characterized in humans, each one with a different tissue distribution (50). *P. gingivalis* is the only known periodontal pathogen to produce PAD (40). The unique ability of *P. gingivalis* to produce PAD provides an association link between periodontal infection and RA (50). In a recent systematic review evaluating serum antibody levels against *P. gingivalis* in patients with or without RA, it is indicated that RA is often accompanied by the presence of an immune response against *P. gingivalis* (51). Thus, the formation of citrullinated proteins in the periodontium in a similar way to those formed in RA, may lead to think that the citrullination induced by periodontitis may play a role in the etiology of RA (52). Additionally, the finding of extra-articular citrullination and the production of ACPAS in periodontal tissues has been repeatedly reported. The presence of mRNAs for PAD-2 and PAD-4 has been found in gingival tissues in the presence and absence of inflammation, while anti-cyclic citrullinated protein antibodies are more frequent in the crevicular fluid of patients with periodontitis. Citrullination processes in the periodontal stroma is dependent on inflammation whereas citrullination in

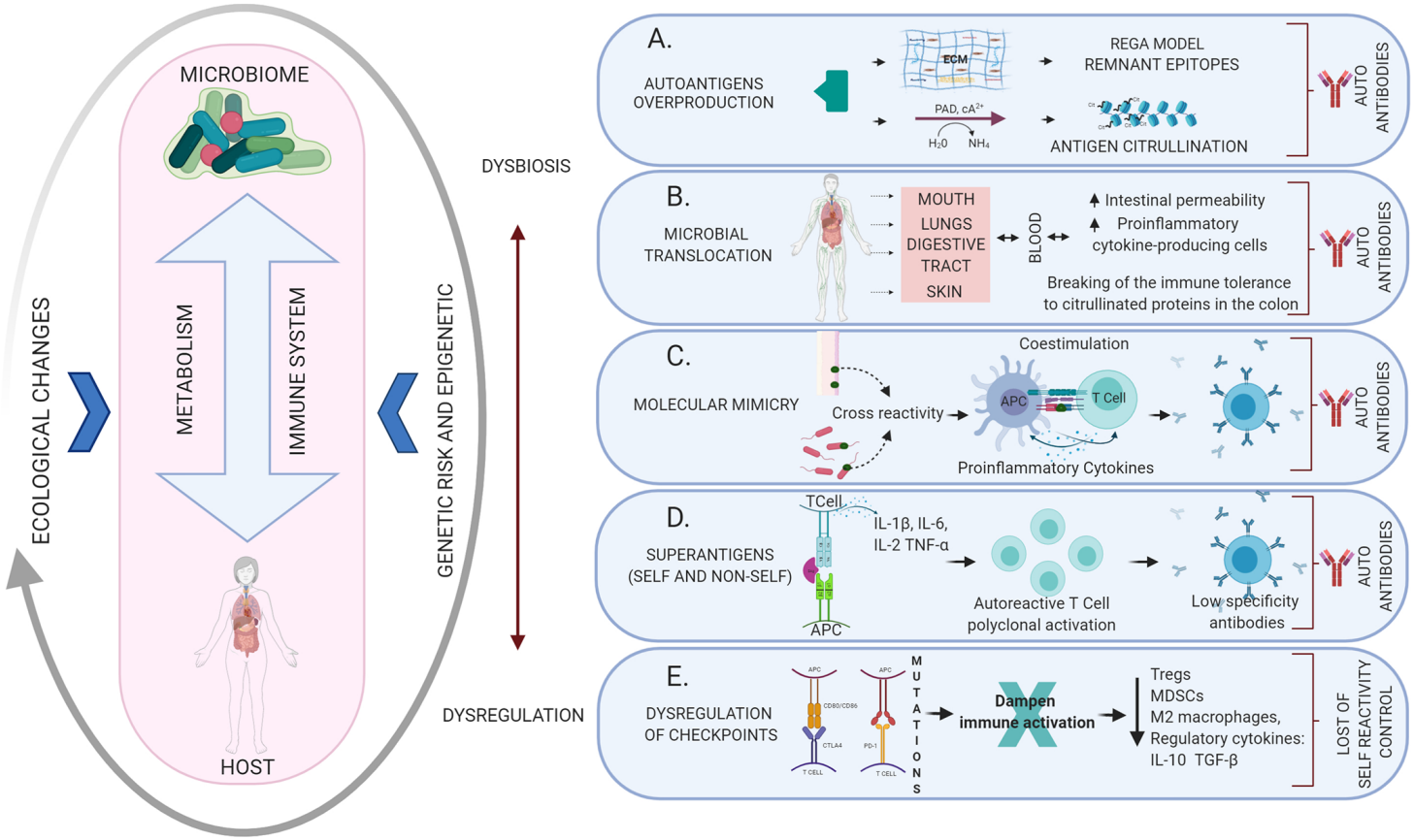

FIGURE 2 | Mechanisms of autoimmunity triggered by dysbiotic changes and dysregulation of the immune system mediated by altered microbiome-host relationships. The host-microbiome interaction may be influenced by several ecological changes (eating habits, aging, infection, among others) that may generate dysbiosis with the consequent activation of different local or systemic autoimmune mechanisms. **(A)** Autoantigens overproduction: the result of the breakdown of the extracellular matrix creating "remnant epitopes" that act as autoantigens or because of enzymatic modification of antigens (citrullination). **(B)** Microbial translocation: bacteria, their products or even products of immune activation against them, can migrate through the bloodstream to different organs leading to an alteration of the local inflammatory response and the production of autoantibodies. **(C)** Molecular mimicry: cross immune response against self-antigens induced by microbial epitopes similar in composition or structure. **(D)** Superantigens: Self or non-self-superantigens interact with the Vβ variable region of the TCR activating and causing T cell expansion. This is a low specificity recognition, which generates low-affinity antibodies that easily end up activating autoimmune mechanisms. **(E)** Dysregulation of checkpoints: alteration of the CTLA4 and PD-1 autoreactivity checkpoints in T cells, resulting in the loss of physiological modulation or regulation of the autoimmune response.

the gingival epithelium has been described as a physiological process (52–54) (**Figure 1A**).

Microbial Translocation

Body circulation is a closed system, actually, blood in healthy organisms was thought to be a "sterile" microenvironment (e.g. in the sense of absence of cultivable microorganisms) and bacteremia could be potentially life-threatening. However, the presence of a blood microbiome has been associated with diseases classified as non-infectious and it has been described that a large number of bacteria could use some alternatives as dissemination mechanisms including intracellular persistence (*Listeria monocytogenes, Salmonella typhimurium*, and *Yersinia pestis*), or using circulating neutrophils as Trojan horses (*Staphylococcus aureus*) (26).

Bacteremia leading to microbial translocation is a viable mechanism in all those systems in contact with the external environment, such as upper and lower respiratory tract, skin, digestive tract, and the oral cavity, from where not only the microorganisms can pass into the blood but also reach the intestine and induce multiple immune responses. Increased intestinal permeability, from where microorganisms or their metabolites could also reach the circulation, has been confirmed as a crucial mechanism in multiple autoimmune diseases (55) (**Figure 2B**).

RA is one of the clearest examples to explain bacterial translocation through different routes associated with alterations in the microbiome (both oral and intestinal) and the occurrence of autoimmunity (56) The intestinal microbiome has been proposed as an indispensable factor in the progression of RA (57). Citrullinated peptides have even been found in the colon, the lungs and synovial fluid of rheumatoid arthritis' patients, supporting the idea that the mucosa of the colon can be one of the breaking sites of immune tolerance to citrullinated proteins (58). The role of the microbiota in the appearance or exacerbation of arthritis has been demonstrated in murine models (59) in which by inducing alterations in the intestinal microflora, production of proinflammatory cytokine-producing cells can be assessed at the intestinal and extraintestinal levels (60). From there they migrate to peripheral lymphoid tissues activating inflammatory processes that in turn result in systemic differentiation of B cells and the production of antibodies, which could trigger the disease *via* recognition of molecular patterns of the intestinal microflora (61).

Beyond RA and diabetes, increased intestinal permeability has been confirmed in inflammatory bowel disease (62) and other autoimmune diseases such as MS. *Helicobacter pylori* (a gastric microorganism) was found in kidney biopsies of patients with lupus nephritis (63), and antibodies against it react with extra-gastric tissues as glomerular capillary walls, ductal, renal tubular cells and glomerular basement membrane (64) for what they have been involved in the pathogenesis of different kinds of nephropathies considered immune complexes mediated diseases. This shows that in some way there is translocation of microorganisms, their components, their metabolites or immune products against them to local tissues, supporting their participation in the occurrence of autoimmunity, although the exact mechanisms of this relationship must be identified.

A recent experiment in which *P. gingivalis* was administered orally to C57BL/6 mice showed changes in the intestinal microflora, the barrier function and the immune system, which is reflected in an increased risk of systemic diseases characterized by, among others, low-magnitude systemic inflammation (65). Translocation is part of these mechanisms since bacterial periodontopathic deoxyribonucleic acid (DNA) has been found in various organs and tissues (66) as well as an increase in interleukin 6 (IL-6) in the bloodstream (67). The oral-intestinal connection has been also proposed as one more hypothesis to establish the link between periodontitis and systemic disease. In animal models, it has been shown that oral administration of *P. gingivalis* can induce intestinal dysbiosis (68) and result in endotoxemia and inflammation of the liver and adipose tissue, as well as an increase in the severity of collagen-induced arthritis mediated by an increase in T helper 17 cells (Th17) function at the intestinal level (65) (**Figure 1B**).

It has also been described in animal models of type 1 diabetes, that oral administration of *P. gingivalis* alters the intestinal microflora, inducing dysbiosis manifested in increased genera *Brevibacterium*, *Corynebacterium*, and *Facklamia*, aggravating glycemic control. In addition to the presence of microorganisms in the feces, an alteration of intestinal permeability was observed (due to action on tight intercellular junctions), as well as a dysregulation of the inflammatory response and glucose-fatty acid metabolism in the ileum and the liver (69). Thus, the translocation of oral microorganisms (including periodontopathogens) (69) and their products including citrullinated peptides in the case of *P. gingivalis* (48) is a recurrently cited mechanism in the interrelation between periodontal diseases, autoimmunity and other inflammatory diseases.

Molecular Mimicry

Molecular mimicry occurs when a self-antigen is so similar to an antigen from a microorganisms or other source in the environment that antibodies raised against that epitope will also bind to the autoantigen. It has long been clear that pathogenic microorganisms can potentially provide ligands that activate cross-reactivity with host-specific antigens (70, 71), which today is established as one of the most accurate links with the generation of autoimmune processes mediated by infections (72), but has also being recognized for a long time as a mechanism of evasion of microorganisms to the direct immune response (73, 74) (**Figure 2C**).

For homologous or even identical peptides to activate that T cell response, they must be available to be presented by antigen presenting cells (APCs) and must be properly processed by proteases and the machinery that performs this function. It is not unthinkable that the reduction of microbial diversity associated with inflammation and autoimmune diseases (75, 76) creates conditions under which microorganisms or their products can, with greater efficiency, traverse the epithelial barrier and be seen by APCs. Strong evidence is needed to demonstrate the role of commensals as antigen providers for

these cross-reactions, to determine whether or not the role of molecular mimicry by commensal bacteria is related to the occurrence of autoimmunity (77).

There are multiple examples that prove the existence of molecular mimicry in the generation of autoimmunity related to different microorganisms, like: heart disease related to a chlamydia infection (78), MS due to infection with a virus in early stages of life that shares antigenic structures with tissues of the central nervous system such as myelin basic protein, inducing demyelination (79); patients with type 1 diabetes mellitus due to the homology in the sequence between the enzyme glutamate decarboxylase (GAD65) of the β cells of the pancreas with the enzyme P2-C related to the replication of the coxsackie B virus (80); rheumatic fever due to cross reaction between bacterial molecules: protein M (higher virulence factor of group A for *Streptococci*) and N-acetylglucosamine with myosin present in the myocardium (81); autoimmune uveitis (82), ankylosing spondylitis (83), Sjogren's syndrome (84), systemic lupus erythematosus (SLE) (40), etc.

The oral cavity, especially in the presence of periodontal diseases, has proven to be a source of antigens both locally and systemically and molecular mimicry is undoubtedly one of the mechanisms by which it participates as a chronic infection in local and remote autoimmunity. The heat shock proteins (HSP) belong to a family of proteins conserved in prokaryotic and eukaryotic organisms during the evolutionary process, whose homology gives them a strong immunogenicity. In a chronic infection the immune response generated by human HSP60 and its bacterial equivalent, activates human vascular endothelial cells for the expression of E-selectin, intercellular adhesion molecule-1, and the vascular cell adhesion molecule-1. This generates the activation of endothelial cells, smooth muscle cells, and monocytes/macrophages for the production of IL-6 and tumor necrosis factor-α (TNF-α) (85).

High levels of human HSP can have a "toxic" effect on the immune system by stimulating pathological forms of activation as in the case of chlamydia infections and this has been defined as "immunovirulence." The periodontopathogenic bacteria of the red complex have HSP homologous to GroEL from *Escherichia coli*. In other bacteria apart from them, such as *Agregatibacter acinomycetemcomitans*, the production of these HSP have been reported. Two subfamilies of proteins, GroEL and Dnak, are present in these bacteria in different amounts and molecular weights (86). The GroEL homologs are known as key molecules for autoimmune type reactions because of their similarity in sequence with human proteins, specifically with the human HSP60 protein present not only in periodontal cells but also in endothelial and muscle (87).

Multiple peptides from GroEL show more than 60% identity with human HSP60 (88) (**Figure 1C**). Antibodies anti-*P. gingivalis* GroEL HSP60 and to human HSP60 (indicating cross-reactivity) are detected in all samples of gingival tissue extracts from periodontitis and periodontally healthy subjects, but a higher frequency of seropositivity and a stronger reactivity is found in the periodontitis patients (64). Also, the existence of accumulated HSP60-specific T cells has been demonstrated in the gingival lesions of patients with periodontitis, with a strong proliferative T-cell response to human HSP60 in periodontitis patients compared with periodontally healthy control subjects. On the other hand, the proliferative response to *P. gingivalis* GroEL was much lower than that to human HSP60, which may indicate that *P. gingivalis* GroEL stimulates regulatory T cells (Tr cells), which play a critical role in the generation and maintenance of tolerance (89). The fact that even healthy patients were seropositive could indicate that these antibodies could act as a beneficial protective immunity to microbial HSP60 acquired by infection.

IgA-HSP60 levels are lower in periodontitis patients, but immunoglobulin G (IgG) class antibodies have been linked to antibody levels for *A. actinomycetemcomitans* and *P. gingivalis* (90). On the other hand, the levels of antibodies against human HSP60 and *P. gingivalis* GroEL in patients with atherosclerosis are significantly higher due to the presence of cross-immunological reactions, which would possibly facilitate endothelial pathology. This indicates that anti-*P. gingivalis* GroEL antibody can react against HSP60 expressed on injured endothelial cells. Antibodies elevated against HSP60 due to periodontal disease, may eventually become a risk factor for developing atherosclerosis in susceptible patients (91). Then, at the systemic level, the possibility of relating coronary and cerebrovascular diseases associated with this autoimmune mechanism has also been described, since elevated serum levels of antibodies against human HSP60, during the course of periodontal disease, may have a cross-reaction with gingival tissue, vascular endothelium and smooth muscle or the possibility of deteriorating pre-existing lesions (92) (**Figure 1C**).

Among other examples of molecular mimicry is the I-β2 glycoprotein which is important in the suppression of coagulation; some antibodies against the protein fraction TLRVYK of this molecule are related to thrombotic episodes. Leukotoxin C of *A. actinomycetemcomitans* has protein sequences (SIRVYK) homologous to those of glycoprotein I-β2. In patients with periodontal disease positive for *A. actinomycetemcomitans*, an antibody response was evidenced for the protein fractions SIRVYK of *A. actinomycetemcomitans* and TLRVYK of glycoprotein I-β2, being relatively equivalent values and these two markers, in turn, higher in patients with periodontal disease regarding healthy patients (93). Under this concept, it was hypothesized that a chronic *A. actinomycetemcomitans* infection can increase thrombogenic antibodies against the I-β2 glycoprotein antibody by molecular mimicry, especially the IgG2 anti-SYRVYK response (94). In addition to the above, *P. gingivalis* also has a protein sequence (TLRIYT), as well as *T. denticola* (TLALYK), that have high homology to the I-β2 glycoprotein peptide, which may be another mechanism of molecular mimicry (95) (**Figure 1C**).

It has also been observed that anti-cardiolipin antibodies (aCL) from patients with periodontitis can be proinflammatory, promoting the activation of cell TLR4 pathways. These antibodies appear to be produced by molecular mimicry given their similarity to antigens of oral bacteria *P. gingivalis*, *A. actinomycetemcomitans*, and *T. denticola*, since all 3 bacteria have antigens with peptide sequences with significant homology to a cryptic binding site. Antibodies in serum to β2GPI protein, the target antigen of aCL,

could indicate that circulating aCLs can induce or influence inflammatory responses at sites distant from the oral cavity (96).

Gingipains from *P. gingivalis* have been reported to be recognized by natural IgM and by being molecularly identical to the epitopes of anti-oxidized LDL (oxLDL) in malondialdehyde-modified LDL. Therefore, it may be that periodontal pathogens stimulate atherogenesis by activating autoimmune responses due to similar antigenic structures in the host, such as HSP or OxLDL, by molecular mimicry (97). After an immunological analysis to show the systemic levels of antibodies against *A. actinomycetemcomitans*, *P. gingivalis*, HSP 60, 65 and 70 and the OxLDL in plasma, it was found that the levels were higher in patients with periodontitis, however none of these markers of molecular mimicry decreased after periodontal treatment, even six months later. Thus, it can be thought that the increased risk of cardiovascular disease in periodontitis patients is associated with a complex and persistent immune response that may become resistant to periodontal therapy (98).

There are other related HSPs. Anti-HSP65 antibody levels have been reported in carotid atherosclerosis and coronary heart disease. Hypertension has also been associated with B cell activation and autoantibody production (anti-Hsp70, anti-Hsp65, anti-Hsp60, anti-AT1R, anti-α1AR, and anti-β1AR) (99).

Thus, molecular mimicry goes beyond the similarity of structures between the host proteome and the microbiome and includes genetic, environmental factors and alterations that are related to the selection processes of T cells in the thymus that allow the filtration of self-reactive cells (100), which makes this mechanism an interesting therapeutic target that is already being explored (101).

Superantigens

Microbial superantigens (SAg) induce the activation and expansion of a large number of T and B cells, which can result in the production of great amounts of regulatory and effector cytokines, modifying the host's immune functions (102). They are mainly secreted by gram-positive bacteria and are capable of inducing pathological symptoms (103). This SAg binding mechanism activates a large proportion of T cells (up to 25% of all T cells) in comparison to conventional antigens (0.0001% of all T cells) (6, 7). SAg differs from regular antigens because interacts with the variable region of TCR-Vb chain and CMH-II in the antigen-presenting cell in a segment outside the antigen-binding site and induce the cell activation by cross linking of receptors (104). Therefore, SAg recognition by T cells has a significantly lower degree of specificity, as compared with their interaction with conventional antigens (105) (**Figure 2D**).

Findings from the late 1990s showed that T cells in periodontitis have a limited repertoire of expression of T cell receptor (TCR) variable region (V) gene products compared to those found in autologous blood which suggested that gingival T cells are not randomly mobilized from peripheral blood and that local events influence the T cell recruitment and expansion repertoire (106). In the gingival tissue of patients with periodontitis but not in healthy tissue, about 50% of all T cells express one or a few families of TCR Vb, leading to the hypothesis that the expansion of T cells in inflamed gums may occur through superantigens present in periodontopathic bacteria (107) (**Figure 1D**).

However, the results seeking to confirm the role of superantigens in associated bacteria and periodontal disease in the inflammatory response were controversial. Mathur's study in 1995 showed that co-culture of peripheral blood mononuclear cells with *Prevotella intermedia* induces expansion of peripheral blood T cells expressing Vab2, Vb5, and Vb6, in patients with periodontitis and healthy individuals, a result that was not found for *A. actinomycetemcomitans* and *P. gingivalis* (108). These findings were confirmed by the expansion of Vb8, Vb12, and Vb17 cells in response to a strain of *Prevotella intermedia* (109). On the contrary, a study carried out in a murine model in which the proliferation of T cells in response to extracts of *A. actinomycetemcomitans, P. gingivalis, Prevotella intermedia* and *Prevotella nigrescens* showed that the extracts of these bacteria are not capable of activating T cells by means of superantigens (110). The role of T cell activation by superantigens has not been demonstrated for *P. gingivalis* that induces expansion of CD4 and CD8 Vb5.2 T cell clones but this activation appears to be antigen specific rather than by superantigen activity (111). In 2005 a review on the aspects of adaptive host response in periodontitis emphasizes that even though autoimmune reactions are evident in periodontitis lesions, the role of superantigens in periodontitis is unclear (112).

Even when in periodontitis the hypothesis of activation by superantigens and their ability to alter the response could not be demonstrated and was abandoned, the mechanism is still relevant to explain the pathogenesis of diseases where the host microbiome interaction is crucial, such as the autoimmune sequelae of streptococcal diseases, toxic shock syndrome with its characteristic dysregulated cytokine storm, allergic inflammation, and tonsillar hyperplasia, among others where bacterial infections can mimic autoimmunity (113–116).

Activation and Inhibition of Receptors Related to Autoimmunity

Overregulation of inhibitory receptors (IR) such as CTL4 (cytotoxic T lymphocyte–associated protein 4) and PD-1 (programmed cell death protein 1) represents an essential mechanism by which immune responses are controlled to maintain immune homeostasis and prevent autoimmunity. The involvement of these checkpoint mechanisms has also been described in evading anti-tumor immunity, they are recognized as one of the signs of cancer (117) and its blockers are widely studied in antitumor therapy (118). Chronic infections, also induce a variety of immunoregulatory mechanisms such as the production of anti-inflammatory cytokines, activation of regulatory T cells (Treg), and the expression of these checkpoint immune molecules (119).

CTLA4 is expressed on T cells and interacts with CD80/CD86 on APCs limiting T activation and leading to anergy. PD-1 is also expressed in T cells and interacts with PD-L1 and PD-L2 expressed in APCs and tumors by sending a negative message to the T cell that leads it to T cell-exhaustion. Check point

inhibitors have been shown to increase ex-vivo effector T cell responses on patients with bacterial, viral, and parasitic infections, including human immunodeficiency virus, tuberculosis, and malaria, making their study an open door for the development of therapeutics for infection control (120). Check points bind immunity regulatory mechanisms such as Tregs, myeloid derived suppressor cells (MDSCs), M2 macrophages, regulatory cytokines such as IL-10 and transforming growth factor beta (TGF-β) to prevent tissue damage, thus their failure is related to infectious and inflammatory diseases (121) (**Figure 2E**).

Humans with CTLA4 mutations develop widespread immune dysregulation. Antibodies that block checkpoints, also bring adverse effects such as the breakdown of self-tolerance, which highlights its role in the physiological modulation of immune responses and therefore has been used as a target of agonist agents to restore tolerance in the context of autoimmunity (122). In periodontitis, an increase in the expression of CTLA4 on $CD4^+$ T-cells has been found related to the presence of periodontitis and, more recently, the study of these IR has specifically focused on the context of the relationship of possible polymorphisms of the gene that encodes it with susceptibility to specific forms of periodontitis in different populations (123–125) (**Figure 1E**).

Polymorphisms in PD-1 have been linked to susceptibility to autoimmune diseases including SLE, atopy, RA, and progression of MS (126). PD-1 expression in periodontitis has been evaluated to explain the presence of immunosuppression (127) and to be used as a therapeutic target (128). No evidence of their analysis was found in periodontitis in relation to autoimmunity, but the results of the studies open an interesting window. In an ex-vivo murine model, the expression of PD-1/PD-L1 in primed T cells with *P. gingivalis* showed an increase in PD-1 expression in $CD4^+$ T-cells in the presence of *P. gingivalis* activation when compared to controls, but these differences were not found in $CD8^+$ T-cells. This overexpression was higher in competent IL-10 cells (capable of producing IL-10), suggesting increased suppressive activity of the interferon gamma (IFN-γ) response in these cells. The study concluded that in addition to IL-10 production, up-regulation of the PD-L1/PD-1 inhibitory signal may be another mechanism used by *P. gingivalis* to suppress $CD4^+$ T-cell response, possibly contributing to the persistence of the bacteria in the body (129) (**Figure 1E**).

Mechanism of Autoimmunity Dependent on The Host Response

Bystander Activation

An adaptation mechanism of innate immunity has been described as bystander activation. This occurs as a consequence of infected cells which alert and give instructions to neighboring uninfected cells to produce inflammatory mediators either by direct cell to cell contact (mediated by GAP junctions by modulation of connexin) or by paracrine action (soluble signals). This mechanism may allow the immune system to overcome the pathogen's ability to disarm the immune signaling pathways in infected cells. The mechanism has been described in viral and bacterial infections. In addition to communication through microorganisms, cytokines or pathogen-associated molecular patterns, macrophages have been described *in vitro* as releasing active inflammasomes to establish contact (130).

Recent evidence could indicate that there is an underestimation of the role of innate pathogen-associated receptors in T cells (131), since they have been shown to have non-classical activation patterns. That goes beyond the exclusive reliance on antigenic recognition through TCR. Both the γδ T lymphocytes and the mucosal associated invariant T (MAIT) cells, and the conventional αβ $CD4^+$ and $CD8^+$ T-cells in mice and humans express TLRs, demonstrating that the cells of adaptive immunity also use these innate signaling pathways leading to the promotion of T helper cell-dependent inflammation through TLRs (132); this makes us think of these receptors as important regulators of the disease during infection.

This process of activation independent of TCR antigen presentation, also occurs by bystander activation, and was described more than 2 decades ago (133) based on the T cell expansion observed in viral infections. It is a heterologous activation of specific non-antigenic lymphocytes, mediated by indirect signals that favors the inflammatory microenvironment such as costimulatory receptor ligands, cytokines, chemokines, pathogen-associated molecular patterns, extracellular vesicles and microbial particles. Bystander activation occurs in both T cells and B cells, in which the stimulation is independent of the BCR. Cells infected by bacteria or viruses can induce activation of uninfected cells through soluble signals such as cytokines, co-receptor expression (example TLR, CD122, and NKG2D), and intercellular communication mediated by GAP junctions (100).

Lipopolysaccharides (LPS) can activate T lymphocytes non-antigen specific. Furthermore, they can activate dendritic cells by up-regulation of CD86 and IFN-γ production. Innate immunity mediators such as DC and natural killer cells (NK), induce bystander activation of T cells in response to TLRs agonists through the production of IFN-α/β/γ (134) (**Figure 3A**).

Multiple autoimmune diseases are associated with bystander activation: RA, SLE, type 1 diabetes, MS, autoimmune hepatitis, and autoimmune thyroid disease, among others (100). Specifically, for direct activation of T cell signaling through TLRs, there is evidence indicating that recognition of T cells by TLR3, TLR4, TLR7, and TLR9 is related to disease aggravation in models of infection, cancer, and autoimmunity (135).

All the mechanisms described above converge in the bystander activation mechanisms of T cells, since it involves the participation not only of innate receptors but of other factors such as superantigens by polyclonal activation of T cells, which in turn triggers a storm of cytokines. Additionally, molecular mimicry, dual TCR signaling, and virtual memory T cells (136) (which has been observed in experimental animals) are other none classical T cell activation pathways (135). Thus, this mechanism, although without a demonstrated specific involvement in periodontal disease, hypothetically could participate in the pathogenesis of periodontitis from the activation of host responses to dysbiotic changes, which

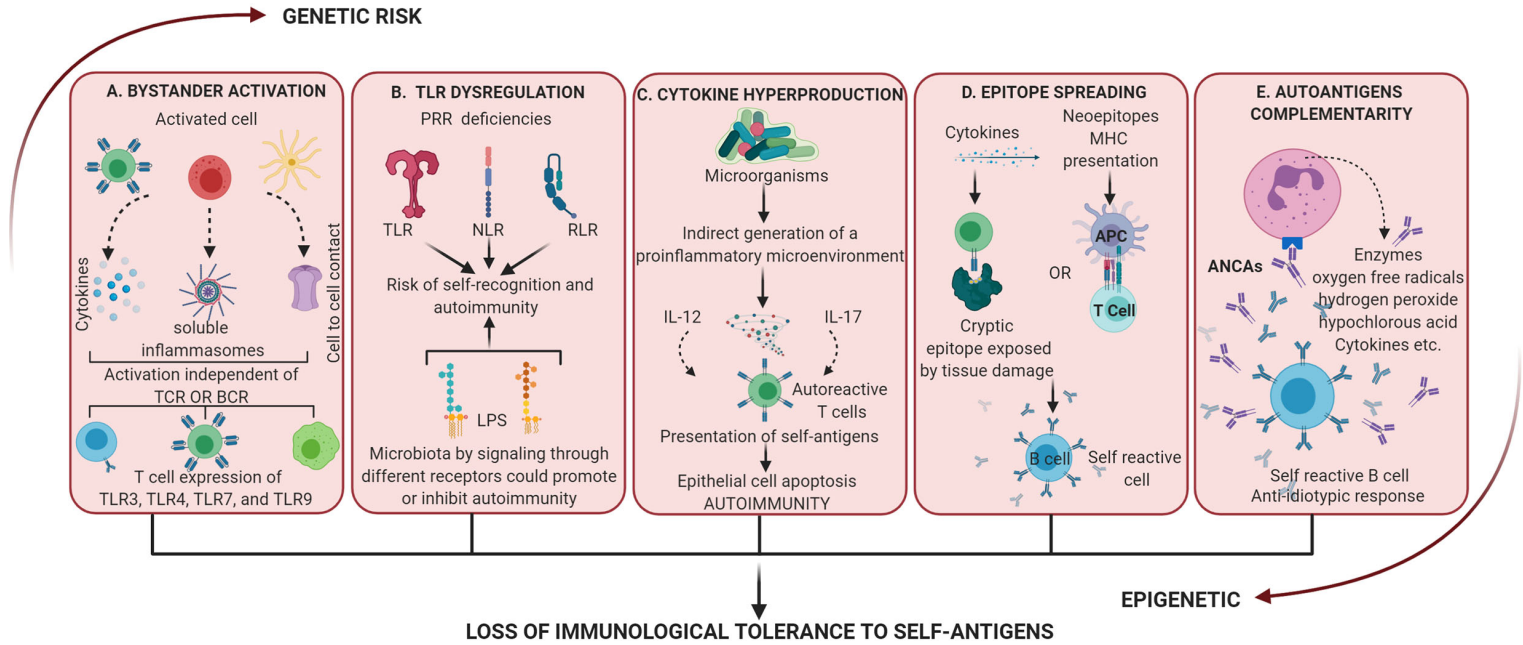

FIGURE 3 | Dysregulation of the activation of inflammatory processes mediated by a dysbiotic microbiota. These mechanisms alone or together lead to the loss of immunological tolerance to self-antigens. **(A)** Bystander activation: an "infected" cell can activate neighboring uninfected cells by cytokine release, the generation of soluble inflammasomes by macrophages, or by cell-cell contact *via* GAP junctions. Bystander activation can induce non-classical activation patterns such as T helper cell-dependent inflammation through TLRs. **(B)** Dysregulation of TLR: these receptors have evolved to sense nucleic acids which comes with the risk of self-recognition and autoimmunity. At the same time different LPS could promote or inhibit autoimmunity by signaling through different receptors. **(C)** Cytokine hyperproduction: proinflammation activated by commensal microorganisms can increase the response of T cells to self-antigen. Also, the differentiation of self-reactive Th17 cells has been related to apoptosis of intestinal epithelial cells induced by microbial stimulation. **(D)** Epitope spreading: it is related to autoimmunity by 2 mechanisms, first inflammation allows T cells to recognize cryptic epitopes and activate B cell complementarity (independent of the antigenic presentation) and second antigen processing and presentation to activate T cell which reciprocally activate B cell (no tissue damage present). **(E)** Autoantigens complementarity: the activation of the autoimmune response does not occur by the autoantigen but its complementary protein-peptide initiating the production of antibodies which activate an anti-antibody response (anti-idiotypic response). ANCAs is an example of autoantibodies generated by these mechanisms and activation of PMNn produces enzymatic and proinflammatory products that result in dysregulation and tissue damage.

involve innate immunity, as in the case of dysregulation of TLRs and all the branches of immunity as in the hyperproduction of cytokines (**Figure 1F**).

Dysregulation of TLRs

Deficiencies in pattern recognition receptor (PRR) signaling that controls homeostasis at the level of composition of the microflora, lead to increased susceptibility to certain diseases (24). When there is inappropriate activation of nucleic acid-sensing, TLRs can cause pathogenic inflammation and autoimmunity (137, 138). TLRs that have evolved to sense nucleic acids and the recognition of microbial DNA or RNA, clearly represents a key strategy by which the innate immune system detects infection; however, the detection of nucleic acids comes with the risk of self-recognition and autoimmunity (139).

For TLR4, a contribution has been suggested in the improvement of autoimmune diabetes in NOD mice, since the deletion of signaling genes as well as the knockout of TLR4 itself have been seen to cause an increase in the incidence of autoimmune diabetes in these animals (140). On the other hand, the deletion of the TLR2 gene reduces the incidence of autoimmune diabetes, suggesting that differences in LPS derived from the microflora between individuals, may prevent the education of their immune tolerance, which would trigger autoimmunity (141). Therefore, the microbiota could promote (through TLR2) or inhibit (through TLR4) the autoimmune responses signaling by different receptors (22) (**Figure 3B**).

The activation of oral epithelial cells, cells of the innate and acquired immune response through CD14, TLR2, and TLR4, are considered a relevant event in the generation of the inflammatory response by the production of proinflammatory cytokines by binding with LPS of periodontopathic microorganisms in periodontal disease. The altered expression of these receptors has been reported in periodontitis (142, 143). TLR 1, 2, 3, 4, 5, 6, and 9 are differentially expressed in connective tissue and epithelium in periodontitis and in greater numbers in periodontitis patients compared to healthy tissue (143) (**Figure 1G**).

Despite these findings, a direct role of TLRs activation as inducers of periodontal damage has not been consistently demonstrated. Just as TLRs expression is found in the inflamed tissue, negative regulatory mechanisms have also been found including the production of soluble forms of these receptors, which inhibit signaling pathways by blocking their ligands (144) (**Figure 1G**). Soluble CD14 levels were significantly higher in gingivitis and periodontitis compared to oral health (145). TLR2 soluble in saliva shows an inverse correlation and TLR4 soluble directly with clinical parameters in periodontitis (146). Additionally, tolerance to the action of endotoxins has been demonstrated by repeated stimulation with periodontopathic bacteria (147). LPS-stimulated cells of the human periodontal ligament have decrease expression of TLR2, TLR4, IL-6, and IL-8 (148).

It has recently been described in a mouse model of periodontitis with RA, that the modulation of cathepsin K-mediated TLR9-related autophagy, can decrease bone destruction in periodontitis promoted by RA, by modulating the infiltration of macrophages, TLR9, autophagy proteins (TFEB and LC3) and inflammatory cytokines (149) (**Figure 1G**).

Collectively these findings show that TLRs in the periodontium and their regulatory mechanisms can be activated or inhibited by specific ligands derived from periodontal bacteria (150). Therefore, a specific role between bacteria or its genetic material and the innate host response by TLRs that leads to the occurrence of autoimmune responses has not been established but cannot be ruled out.

Amplification of Immunity by Hyperproduction of Cytokines

The amplification of immunity by hyperproduction of cytokines was related to the appearance of autoimmune responses after discovering that the production of proinflammatory cytokines activated by commensal microorganisms (specifically IL-12 at the level of gut-associated lymphoid tissues) can increase the response of T cells to self-antigens (22). IL-12 is attributed a dual role in periodontal disease; in gingival fluid (151, 152), gingival tissue and serum (153), it has been associated with the severity of periodontal destruction. Additionally, the polymorphism of the IL-12 gene is associated with susceptibility to the development of chronic periodontitis (154) and studies in IL-12 deficient mice with *P. gingivalis*-induced periodontitis show less bone resorption which supports the participation of this cytokine in the development of bone destruction in the disease (155). On the other hand, IL-12 induces the expression of immunosuppressive molecules, such as human leucocyte antigen (156) and indoleamine-pyrrole 2,3-dioxygenase (157) through an IFN-γ–dependent pathway. IL-12-induced immunomodulation may be an important mechanism that helps regulate the host's immune response and maintain tissue homeostasis during periodontal inflammation. These findings suggest positive and negative influence of IL-12 on periodontal disease (158). However, it is not known how dysbiosis of the periodontal microbiota can guide IL-12 function towards a protective role or proinflammatory function. Understanding the role of the microbiota in this mechanism could be used to regulate or control the evolution of periodontal disease.

Despite the regulatory processes in which cytokines participate, microorganisms are generally related to autoimmune responses by indirect generation of a proinflammatory microenvironment where this expression of cytokines would favor the presentation of self-antigens leading to autoimmunity (17). Apoptosis of intestinal epithelial cells in response to microbial stimulation has been shown to increase the presentation of self-antigens resulting in differentiation of self-reactive Th17 cells (159). Although this mechanism has not been specifically described for periodontopathogens, the tropism of the pathogen could determine the specific location of the inflammatory disease (**Figure 3C**).

It has been studied in various germ-free animal models how the production of cytokines by cells of the innate response such as DC, macrophages, neutrophils and NK after their activation by microorganisms, are necessary for the development of

autoimmunity. For example, macrophages isolated from germ-free mice release lower levels of TNF-α and higher levels of IL-10 when stimulated with bacterial LPS (160). NK function is also altered under these conditions, possibly due to the inability of monocyte-derived cells to produce type I interferons in response to microbial stimuli (77). Additionally, the decrease in the number of Th17 cells in the intestine in germ-free mice improves autoimmune glomerulonephritis (22).

IL-17 is of particular interest in the pathogenesis of periodontitis because it is involved in both the inflammatory response and protective immunity against microorganisms (161, 162). Constant signaling through the IL-17 receptor can transform a protective acute inflammatory response into a chronic immunopathological response (163).

In periodontal disease, both IL-17 and IL-17F activate cells of innate immunity as neutrophils and cells from surrounding non-immune tissues (fibroblasts and epithelial cells), by activating the transcription factor NF-κB. IL-17 can activate both fibroblasts and endothelial cells to increase secretion of IL-6 in the presence of TNF-α (**Figure 1H**). IL-6 and RANTES are recognized factors for the progression of gingivitis to periodontitis. RANTES is produced by gingival fibroblasts after challenge with IL-1β and TNF-α, inducing recruitment of monocytes in the tissue; it is also a chemo-attraction factor of Th1 profile cells. The concentration of IL-6 and RANTES is high in deep (≥6 mm) periodontal pockets (164).

Although the mechanisms are not clear, there is evidence to suggest that *P. gingivalis* promotes an IL-17 rich environment, as a mechanism for obtaining nutrients that come from the degradation of oral tissues caused by inflammation (165). The Th17/Treg ratio in autoimmunity and Th17-mediated immunity is important for maintaining hematopoietic and mucosal homeostasis. Altered homeostasis between Th17 and Treg has been implicated in several autoimmune diseases, so the relationship between Th17 effector cells and Treg must remain in balance to preserve functional immunity and host health (166). Additionally, the trans-differentiation of Treg cells into cells that produce IFN-γ similar to Th1, and from Th17 cells that produce IL-17 to IFN-γ, has been implicated in the pathogenesis of several autoimmune diseases (167–169) (**Figure 1H**). Such trans-differentiation process has been demonstrated in a mouse model inoculated orally with *P. gingivalis* at 4-day intervals to simulate persistent dysbiosis. Results showed that in this environment of persistent dysbiosis as occurs in periodontitis, the $CD4^+$ T lymphocyte-mediated immune response evolves from one initially dominated by IL-17A to one that is predominantly IFN-γ-producing, in a response generated *de novo* by Th1 cells. A small proportion of Treg cells expressing IL-17A on day 28 disappear on day 48. This evolution of dysbiosis and the inflammatory environment on day 48 by *P. gingivalis*, induces a transdifferentiation of Th17 and IFN-γ expression. The components of the microbial biofilm or host cells under the influence of such a microbial environment responsible for driving Treg or Th17 transdifferentiation in the oral environment are not known (170). Changes in microbial communities from the initiation of an accumulation and conformation of non-specific oral biofilm to subsequent dysbiotic changes, rather than a simple increase in microbial load, are necessary for the activation of disease-causing Th17 cells in mice and in humans (171). Therefore, transdifferentiation mechanisms may contribute to the transition from active inflammation to a resolution phase at sites with periodontitis.

Epitope Spreading

In many immune responses the initial antigens and epitopes of those antigens that are recognized by adaptive immunity are limited. Over time, the responses of T and B cells can grow to include many other epitopes and additional antigenic molecules, this process is known as epitope spreading. Under normal conditions, by expanding the antigenic epitopes recognized by the immune system, the response to these foreign antigens is optimized, allowing neutralization *via* antibodies, recognition by various immune cells, and clearance of the pathogen (172). The epitope spreading is essential for the development of normal adaptive immune responses, but in turn contributes to the immunopathological processes of infection-induced autoimmunity.

There are 2 mechanisms for the epitope spreading to occur in autoimmunity, one independent of the antigenic presentation, in which inflammation and cytokine activation are sufficient to allow T cells to recognize cryptic epitopes and activate B cell complementarity (173), and the other dependent on binding to an APCs that occurs when there is no tissue destruction, and relies on processing and presentation to activate T cell which in turn reciprocally activate B cell (174). Molecular mimicry and cross reactivity B cell epitope spreading after mimicry, is a mechanism by which the body initiates the breakdown of self-tolerance, leading to the development of autoimmune diseases. More is known about T cell spreading epitope than B cell epitope in disease (172). Therefore, it all begins with molecular mimicry against the dominant epitope, resulting in an auto-response directed against a neo-epitope. Intra-intermolecular epitope spreading is one of the four mechanisms on how antigens of infectious organisms may propagate into various human autoantigens to trigger adaptive autoimmune responses. Substantial evidence supports the hypothesis that tissue damage can lead to epitope spreading, which can then contribute to ongoing disease (175) (**Figure 3D**).

The peptide 19 from *P. gingivalis* HSP60 (Pep19) has been studied as one of the dominant peptides, from which, the epitope-specific immune response to subdominant epitopes could be sequentially diversified towards autoimmune responses directed against human neoepitope in periodontal disease induced by *P. gingivalis* and in autoimmunity (91). The mechanism as Pep19 directs the epitope spreading towards the formation of autoantigens in chronic periodontitis or in experimental periodontitis induced by *P. gingivalis* is not clear. Animal and human model research has described that in both young, systemically healthy humans and mice, there is a unique dominant immune response against Pep19, which persists in the presence of chronic periodontitis and autoimmunity, without subsequent replacement by response to subdominant epitopes (87, 174). Pep 19 generates sequential spreading epitope to Pep19

subdominant autoantigens of human HSP60 (Hu19) in healthy subjects and mice, to P9 autoantigens of human HSP60 (Hu9) and to oxidized low-density lipoprotein (ox-LDL) (neoantigen) in the Periodontitis induced by *P. gingivalis* as well as in autoimmunity. The proliferative activity of T cells to the autoantigens Hu19, Hu9 and ox-LDL, and the cross reactivity of Pep19 monoclonal antibodies to these epitopes are proposed as the responsible cellular and molecular mechanisms (175) (**Figure 1I**).

Pep19 has also been the object of study for immunotherapy as a target of Treg cell subsets in search of generating tolerance. Naive cells $CD4^+CD25^+CD45RA^+$ Tregs and the epitope spreader Pep19 were proposed as cell and molecular targets for a scheme of antigen-specific Tregs-based vaccination in collagen-induced arthritis (177).

Autoantigens Complementarity

The theory of autoantigen complementarity (described in the 1990s) is based on the fact that the autoantigen is not the one that triggers the events that lead to autoimmunity, and that what activates it is its complementary protein-peptide (178). This initiates the production of antibodies which in turn activate an anti-antibody response or anti-idiotypic response. The anti-idiotypic antibody reacts with the autoantigen which has a sequence complementary to that of the initiator antigen. This mechanism was described from the study of cytoplasmic anti-neutrophil antibodies (ANCAs) directed against proteinase 3 (PR3) of neutrophil granules (179). It was discovered that patients not only had antibodies against PR3 but antibodies against peptides encoded by the noncoding band of genes encoding the PR3 autoantigen (178). This means that the initiator of the autoimmune response is not the autoantigen but rather the complementary protein in the microorganism that induces the idiotypic antibody response (**Figure 3E**).

ANCAs are defined as autoantibodies of the IgG type mainly directed against granular components of the neutrophil, specifically on proteolytic enzymes located within the azurophil or primary granules. Initially, their production was attributed to the polyclonal activation of autoreactive B lymphocytes but now they are considered as antibodies generated by autoantigens complementarity (180). When the binding between the ANCAs and target antigens occurs, an activation signal is emitted to the neutrophil for the release of enzymatic products (collagenases, elastases), oxygen free radicals, hydrogen peroxide, hypochlorous acid, hydroxyl radicals and nitrous oxide; cytokines and pro-inflammatory substances such as: IL-1β, TNF-α, platelet activation factor, thromboxane E2, and leukotrienes. This activates autocrine and paracrine responses to other cell types without regulation, added to the inactivation of enzymatic inhibitors, resulting in evident cell damage (181).

The apoptosis of neutrophils and their subsequent tissue removal is a vital function that must be fulfilled in order to not break the balance as apoptosis of neutrophils is essential for controlling the duration of early inflammatory response and thus limiting the local tissue damage that can result from prolonged activation of neutrophils. Defects in apoptosis or in the process of removal of apoptotic cells could lead to exposure of these cellular fragments to immune system and activating a humoral immune response (182). Thus, another mechanism by which the ANCAs exert their function is by preventing apoptosis and the removal of apoptotic neutrophils, generating a sustained immune response (183). This is a topic that has recently gained strength to explain the pathogenesis of autoimmune diseases characterized by a defect in apoptotic cell clearance and in the resolution of inflammation (i.e. granulomatosis with polyangiitis, an autoimmune necrotizing vasculitis associated with ANCAs), where PR3 that acts as an autoantigen impaired C1q enhancement of apoptotic cell uptake by altering the elimination of apoptotic bodies (184).

Initially described in systemic pathologies such as RA (185–188) and SLE (189–191), towards the end of the 1990s ANCAs were related to periodontal disease, due to the relationship established between these diseases and periodontitis. Novo et al. would be the pioneers in mentioning this mechanism in periodontal disease visualizing p-ANCA immunofluorescence patterns (191).

In the periodontal environment, ANCAs also seems to interfere directly with the apoptosis of neutrophils present in the lesion (192) (**Figure 1J**). When the oxidation of α-1-trypsin (inhibitor) occurs, this leaves free Proteinase 3 to be targeted by the enzyme attack. As a result, there will be high levels of substances and protein products mediating periodontal damage in the environment, such as: collagenase, gelatinase, cathepsin B and D, elastase, β-glucuronidase, myeloperoxidase, and lysozyme (**Figure 1J**). In addition, a greater number of molecules will be found, such as adhesins, selectins, and integrins that will allow greater adhesion to the vascular endothelium. Even so, it has been described that in early stages of periodontal disease when there is no polyclonal activation of B cells there is no presence of ANCAs (193).

In 2006, 15 years after the beginning of the study of ANCAs in periodontitis, a model was proposed to explain the pathogenesis of periodontal disease from the concept of ANCA associated autoimmunity. Based on multiple studies, the authors founded their model on the following points: 1. Genetic factors similar for periodontal diseases and other ANCA-associated diseases like Wegener's granulomatosis, RA and, to a lesser extent, LES; 2. The activation of the cells bearing the ANCA antigens (neutrophils and monocytes) leading to an inflammatory response and eventually resulting in immense bystander damage; 3. The damage to other cells bearing the target antigens such as endothelial cells; 4. A loss of function by the direct inactivation of the antigen to which the ANCA bind; 5. The induction of the respiratory burst and the degranulation of neutrophils mediated by ANCA with the consequent release of enzymes and various metabolites capable of producing damage to the periodontal connective tissue; and finally 6. The local or systemic presence of superantigens that could ensue from periodontal pathogens and that may stimulate monocytes/macrophages to secrete proinflammatory cytokines and elevated levels of TNF-a reported in $ANCA^+$ patients. The publication concludes that even though the evidence supporting the model puts

periodontitis in the spectrum of diseases where autoimmunity mediates damage, further long-term prospective controlled studies were required to validate the ANCA-mediated periodontal destruction model; such studies were never done (183). Despite this type of approach, there is no direct evidence to support the participation of ANCAs in the tissue damage observed in periodontitis.

Relevance of Auto-Antibodies in The Autoimmune Damage

Autoimmune responses are part of multiple inflammatory processes including primary immunodeficiencies, in which the classic sign is the presence of autoantibodies. Autoreactive cells are found in healthy individuals and do not normally cause pathology, this could be due to low precursor frequency or regulatory controls that exceed them; but, in genetically susceptible individuals, regulation of activated autoreactive lymphocytes during tissue damage can be faulty and the pathogen can lead to disease *via* epitope spreading (175). Thus, 2 scenarios can arise: 1. An immune response to a persistent antigen with direct lysis that could cause damage to the own tissues (175); or 2. That healthy individuals having constitutive autoreactive cells indicates that they have a protective role. In fact, B cells produce antibodies that recognize their own epitopes, that have been described as beneficial for their participation in the cleaning of apoptotic bodies and the development and homeostasis of B cells (100) (**Figure 4**).

The study of self-responsiveness in the occurrence of tissue damage linked to the presence of microorganisms is complicated. It has been described that the participation of B-cells in the microbiome host interaction at the intestinal level, goes beyond the promotion of Breg cell differentiation and its production of IL-10 through the activation of IL-1β and IL-6; commensals seem to play a key role in the removal of autoreactive B-cells from the gut associated lymphoid tissue, a process that is defective in SLE and RA, so a lack of regulatory promoting bacteria that compromises synthesis or secretion of immunoglobulins could be hypothesized (40).

Amyloid fibers (curli fibers), a major extracellular matrix protein produced by bacteria and a component of bacterial biofilms, are capable of binding to extracellular DNA and forming curli/DNA complexes that lead to immune activation and the production of autoantibodies (40), establishing an important relationship of bacteria in biofilms with the possible immune dysregulation characteristic of autoimmunity responses.

In periodontal disease, antibodies have been found against almost all types of collagen (I to IV), desmosomal epitopes, aggregates of IgG, host DNA, antigens of the neutrophil cytoplasm, proteins of the extracellular matrix (vimentin, spectrin, filaminin, actin, laminin, keratin, and tubulin) and phospholipids (93, 191, 194, 195). It is possible that due to the

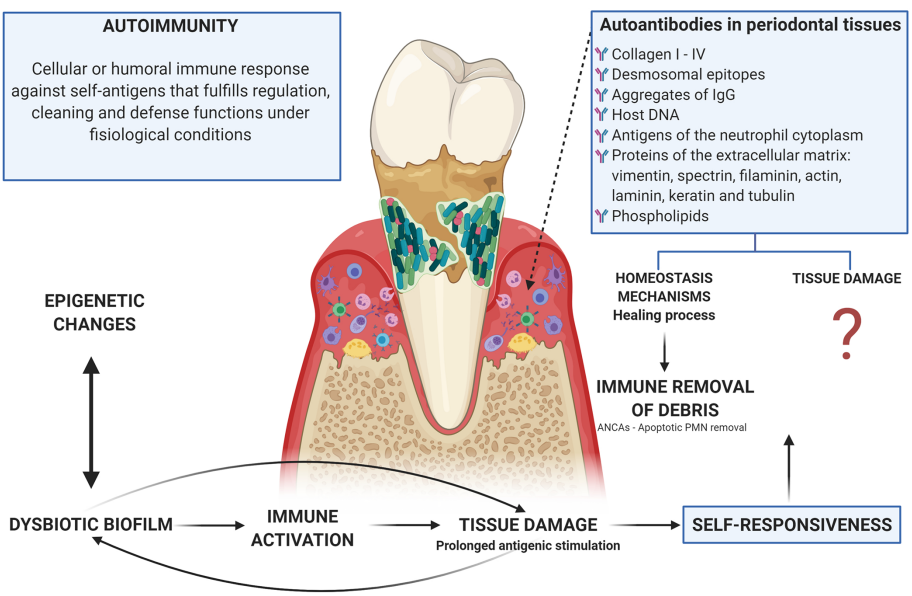

FIGURE 4 | Autoimmunity in periodontitis: tissue damage related or homeostasis mechanisms? There is an evident relationship between the dysbiotic microbiota, the activation of inflammatory mechanisms, and the tissue damage observed in periodontitis, which leads to prolonged antigenic stimulation due to tissue breakdown with the release of matrix proteins. In turn, microorganisms use these products of tissue damage as a nutritional source. This persistence of both self and exogenous (microbial) antigens leads to the occurrence of autoimmunity, which can be understood as a mechanism by which debris generated by tissue damage are removed through the generation of autoantibodies (including ANCAs as a mechanism for the cleaning of apoptotic bodies after the activation of phagocytic cells) or as a damage machinery, although the evidence for the participation of autoimmune mechanisms in the tissue damage observed in periodontitis is poor and lacking analysis. Additionally, microorganisms and their products can alter the activity of epigenetic enzymes at the same time that these epigenetic changes can induce dysbiosis, thus epigenetics being a determining factor in the occurrence of autoimmunity due to a direct relationship with the breakdown of immunological homeostasis.

prolonged antigenic stimulation caused by the rupture of the tissue and the release of the proteins of the extracellular matrix (a secondary event to the inflammatory process induced by microbial dysbiosis), these antibodies that can be considered as part of tissue homeostasis, suffer a change in isotype from IgM to IgG or IgA, while increasing concentration at the site of local inflammation (196, 197). However, IgG anti-collagen type I antibodies are present in gingival fluid during the inflammatory healing process (198), what is opposed to their participation in the damage and supports a possible role for instance in the regulation through what we could call immune removal of debris. Anti-desmosomal antibodies then appear to be a normal part of the immune repertoire (194) (**Figure 4**).

On the other hand, the levels of anti-desmosome antibodies are higher in sites with active periodontitis compared to unaffected sites in patients with periodontitis (194). Similarly, levels of anti-type I collagen antibodies are higher in gingival tissue than in peripheral blood (199), and IgG and IgA isotypes are found in higher concentrations in extracts of gingival tissue than in autologous serum while there are no differences in IgM levels (200). Anti-collagen antibody-producing B cells are found primarily in gingival tissue and in very low amounts in peripheral blood from patients with periodontitis (201). Additionally, serum levels of anti-CD24 IgG antibodies, a surface glycoprotein involved in cell-cell signaling and adhesion in epithelial tissue, have been reported to correlate with a more favorable clinical status in patients with periodontal disease (202).

Finally, one of the immune system functions, related to damage in autoimmunity by the formation of immune complexes, is the complement activation. The relationship between the complement system and autoimmunity appears paradoxical as both the deficiency and the activation, contribute to induce autoimmune diseases (203). The role of the complement system in enhancing infective diseases in secondary complement deficiencies, was mainly demonstrated in patients affected by autoimmune diseases. It has been suggested that the early part of the classical pathway, activated by the immune complex, plays a protective role against the development of SLE, whereas central and terminal component can contribute to disease development (204, 205). Recently, complement also emerged as a critical player in adaptive immunity *via* its ability to instruct both B and T cell responses, it has an impact on T cell responses that now permits to see this system functions also within cells and it´s involvement in regulating basic processes, predominantly those of metabolic nature (206). The presence of complement deposition in affected tissues, decreased levels of complement proteins (207) and high levels of complement activation fragments in the blood and vessels (208) have been documented related to autoimmunity.

Although complement activation has been described as an important mechanism in tissue damage in periodontal and peri-implant disease (209, 210), the most accepted interpretation of its activation is the capacity of the periodontopathogens that use it as a mechanism for tissue degradation in favor of the release of nutritional components, and thus the maintenance of biofilms (211, 212) and not directly as a mechanism of tissue damage.

Epigenetics and Autoimmune Responses

Epigenetics refers to heritable genomic expression without alterations in the original DNA sequence (3) leading to remodeling of the chromatin and activation or inactivation of a gene (213). There have been described three epigenetics modifications: DNA methylation, histone modifications, mainly by short-chain fatty acids and microRNA (miRNA) regulations (3). These processes are potentially reversible and transient. It can be induced or altered by environmental factors that modulate and affect the gene expression and functions (213). Epigenetic regulations could break the immunological homeostasis and result in the development of autoimmune diseases (40) such as SLE, RA (214), Systemic sclerosis (215), Sjogren syndrome (216), etc, among others due to the inflammatory responses mediated by an increase in the number of iNKT, less reactivity of the NK, and alteration of production IFN type I, IL-6, IL-12, IL-18 and TNF-α (22).

SLE is the most studied autoimmune disease correlated with epigenetic modifications, a pattern of robust demethylation of interferon signature supporting a pathogenic role for neutrophils in lupus has been suggested, as well as a model whereby DNA from lupus neutrophils externalized by NETosis enhance type-I IFN production *via* TLR-9 stimulation by hypomethylated DNA (217). Other epigenetic modification described for the SLE are DNA methylation and histone modifications which regulate gene expression in mature T cells for different genes such as CD11a, perforin, CD70, and CD40LG are described in the literature (218), as well as overexpression of IL10 in T cells from SLE patients leading to an increase of specific autoantibody production and tissue damage (219).

Microbial metabolites can affect the activity of epigenetic enzymes or act as necessary substrates for epigenetic modifications. ncRNAs or epigenetic enzymes of the microbiome, can translocate to host cells influencing their gene expression. These epigenetic changes can also induce dysbiosis (40). This leads to the conclusion that the microflora is epigenetically regulated by the expression of genes that buffer autoimmune inflammation but increases the ability to respond to external challenges (22). All exposed responses could be microorganism-specific, depending on host susceptibility and lifestyle factors, as well as varying from animal to human models (**Figure 4**).

It has been established that both genetic and epigenetic factors play indispensable roles in the pathogenesis of periodontal disease (220, 221). Genetic background is necessary for disease onset, but it is insufficient for disease development. Epigenetic modifications also participate in the pathogenesis of periodontitis in genetically predisposed individuals (3) (**Figure 2**).

The post-translational modification of histone proteins in chromatin and the methylation of DNA are the two primary epigenetic mechanisms in periodontitis (3, 213). In addition, many environmental factors may have profound effects on the epigenetic changes and induces susceptibility to disease (3).

DISCUSSION

The recognition of the microbiota role in autoimmune responses has led to greater clarity in patterns of common mechanisms between autoimmunity and primary immunodeficiencies in humans (222). After consolidating the information, the question is: Are autoimmune responses in periodontitis a harmful machinery leading to tissue damage or the dysregulation of the immune mechanisms generated in a dysbiosis-induced inflammatory response, or normal physiological responses to the local presence of microorganisms?

To analyze the occurrence of the autoimmune mechanisms in periodontal disease, it is necessary to start from several facts at the level of periodontal tissues which establish the relationship between periodontitis and autoimmune responses: 1. Presence of microorganisms (commensal or pathogenic) that leads to constant antigenic stimulation by microbe-associated molecular patterns (MAMPs) (223); 2. Prolonged presence of immune activation by damage-associated molecular patterns (DAMPs) (224), given the persistent production of "damage" with a large generation of residues; 3. Constant activation of the innate and acquired immune response, including regulatory mechanisms; and 4. Continuous change in the characteristics of the environment influenced by external factors, including constant passage of microorganisms through the mouth and lifestyles, that trigger the need for uninterrupted epigenetic adaptation (225).

On the other hand, the relatively low prevalence of severe periodontal damage in the population highlight the importance of genetics, mediating not only susceptibility to infection (226), but differences in host response (227) and even susceptibility to the occurrence of inflammatory diseases (228). The 2009-2012 NHANES identified severe periodontitis in 8.9% of US adults (229) and an average worldwide prevalence of severe periodontitis has been estimated to be 11%, including countries with relatively little emphasis on periodontal health care (230). Despite the low prevalence of severe disease, the need to control dental biofilm as the main risk factor in the occurrence of periodontitis should be emphasized, as it has been found that fair to poor oral hygiene increases the risk of periodontitis by two- to five-fold and that the effect of oral hygiene on periodontitis is stronger than those of other risk factors, such as diabetes, smoking or obesity (231).

Autoimmune responses have been described in both periodontally healthy and diseased individuals; but is in susceptible individuals where it is observed that the inflammatory process ultimately results in damage, with a constant altered regulation of the local response. From the data collected, the following assumptions were made based on the analysis of the autoimmune mechanisms occurrence in periodontitis:

Neither the autoimmune mechanisms dependent on the microorganism or those caused by activation and local dysregulation of immune responses by dysbiosis, can explain the occurrence of tissue damage in periodontitis; the studies as presented just support their activation. Superantigens and inhibitory receptors inactivation could not be shown to be related to the occurrence of tissue damage due to autoimmunity in periodontitis; others such as bystander activation that ends in the possible alteration of cytokine production are not clear, as it could eventually be linked to intracellular invasion by periodontopathic bacteria that presents more as an evasion mechanism to generate nutrients for its survival, than to an autoimmune response (232).

Similarly, the role of oral dysbiosis in the induction of the inflammatory response mediated by cytokine hyperproduction is not yet clear, especially since the mechanisms have been proposed from autoimmune diseases as an explanation for the damage caused by the inflammatory response itself (15). However, this does not seem to be the case in periodontitis, since the few studies that use *P. gingivalis* for disease modeling show dual functions for the main cytokines with inflammatory functions in autoimmunity, eg. the hyperproduction of IL-17 of great interest in the pathogenesis of periodontitis due to its involvement both in the inflammatory response and in the protective immunity against microorganisms (233). However, this makes it a double-edged sword in a disease like periodontitis that is initiated by the bacteria, and in which tissue damage is done and controlled by the host's response induced by the microorganism and not by autoantigens since the autoimmune mechanisms in periodontitis seem to be part of the physiological repair process.

In relation to the above and under the exposed premise that autoimmune mechanisms overlap, the role of TLRs dysregulation in autoimmune responses in periodontal disease is controversial and complicated, since there appears to be potential heterogeneity of responses through TLRs mediated by different commensals (150, 234). Thus, the activation of certain TLRs leads to the activation and that of others to the inhibition of the differentiation of cell populations related to autoimmune responses, especially Th17 (235).

The mechanism by which spreaders, such as Pep19, direct the epitope spreading toward autoantigen formation in chronic periodontitis or in experimental periodontitis induced by *P. gingivalis* (175) is unclear. What is deduced from the studies, both in animal model and in humans, is that the response to the spreader is found in healthy individuals and is maintained without being replaced by responses to other epitopes, in chronic periodontitis and even in autoimmunity (87, 176).

Regarding the role of autoantigen complementarity and the increased auto-antibody response found in periodontitis, they have traditionally been proposed as autoimmune responses involved in tissue damage; but in light of new concepts on host-microorganism interactions and microbial dysbiosis in this disease (236), the tissue damage associated with its presence in inflamed tissues occurs more due to the dysregulation of physiological mechanisms guided by a dysbiotic inflammophilic microbiota, and is needed to cover the removal requirements of apoptotic bodies and tissue degradation proteins. The presence of some autoantibodies, in gingival fluid and serum show similar specificity in healthy individuals and periodontitis patients (194), suggesting that autoantibodies are a normal part of the immune repertoire. Therefore, the presence of autoantibodies in

periodontitis does not support their causal role in the occurrence of local tissue damage (**Figure 4**).

In conclusion, in periodontitis, additional consideration should be given not only to the presence or absence of cells with autoimmune functions but to whether or not they are activated and directly related to tissue damage. Despite the lack of evidence to support some autoimmunity mechanisms in periodontitis (which does not mean that they do not participate in the pathogenesis), and after analyzing the physiological functions of such mechanisms in other inflammatory and autoimmune diseases, we hypothesize that the presence of autoreactive cells in periodontal tissues do not explain their direct relationship with tissue loss and may represent the activation of damage "control" mechanisms, as supported by the evidence of activation of the same mechanisms in periodontally healthy patients.

However, the translocation of oral microorganisms, its components or its metabolites from periodontal tissues could be involved in the occurrence of autoimmune responses at a systemic level, in which the different mechanisms mentioned are represented, even if the dynamics exact of this relationship have not yet been clarified. Thus, at systemic level, the link of autoimmunity and periodontopathogens needs to be interpreted from the role of dysbiosis in the occurrence of systemic autoimmune responses, related to the possibility of periodontopathic bacteria ability to generate an alteration of the microflora in other organs with the consequent activation of autoimmunity mechanisms. The processes by which the presence of oral pathogens in remote sites generate dysbiosis in different organs, which in turn dysregulate the systemic immune response in individuals susceptible to autoimmunity, are just being clarified.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Nicholson LB. The immune system. *Essays Biochem* (2016) 60(3):275–301. doi: 10.1042/EBC20160017
2. Kaur G, Mohindra K, Singla S. Autoimmunity-Basics and link with periodontal disease. *Autoimmun Rev* (2017) 16(1):64–71. doi: 10.1016/j.autrev.2016.09.013
3. Zhang P, Lu Q. Genetic and epigenetic influences on the loss of tolerance in autoimmunity. *Cell Mol Immunol* (2018) 15(6):575–85. doi: 10.1038/cmi.2017.137
4. Wong GK, Heather JM, Barmettler S, Cobbold M. Immune dysregulation in immunodeficiency disorders: The role of T-cell receptor sequencing. *J Autoimmun* (2017) 80:1–9. doi: 10.1016/j.jaut.2017.04.002
5. Allenspach E, Torgerson TR. Autoimmunity and Primary Immunodeficiency Disorders. *J Clin Immunol* (2016) 36 Suppl 1:57–67. doi: 10.1007/s10875-016-0294-1
6. Schmidt RE, Grimbacher B, Witte T. Autoimmunity and primary immunodeficiency: two sides of the same coin? *Nat Rev Rheumatol* (2017) 14(1):7–18. doi: 10.1038/nrrheum.2017.198
7. Stappenbeck TS, Hooper LV, Gordon JI. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proc Natl Acad Sci USA* (2002) 99(24):15451–5. doi: 10.1073/pnas.202604299
8. Fagundes CT, Amaral FA, Teixeira AL, Souza DG, Teixeira MM. Adapting to environmental stresses: the role of the microbiota in controlling innate immunity and behavioral responses. *Immunol Rev* (2012) 245(1):250–64. doi: 10.1111/j.1600-065X.2011.01077.x
9. Sommer F, Backhed F. The gut microbiota–masters of host development and physiology. *Nat Rev Microbiol* (2013) 11(4):227–38. doi: 10.1038/nrmicro2974
10. Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* (2010) 10 (3):159–69. doi: 10.1038/nri2710
11. Brandtzaeg P, Kraus FW. Autoimmunity and Periodontal Disease. *Odontol Tidskr* (1965) 73:281–393.
12. Page RC. Gingivitis. *J Clin Periodontol* (1986) 13(5):345–59. doi: 10.1111/j.1600-051X.1986.tb01471.x
13. Hajishengallis G. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. *Trends Immunol* (2014) 35 (1):3–11. doi: 10.1016/j.it.2013.09.001
14. Medzhitov R, Schneider DS, Soares MP. Disease tolerance as a defense strategy. *Science* (2012) 335(6071):936–41. doi: 10.1126/science.1214935
15. Stecher B, Hardt WD. The role of microbiota in infectious disease. *Trends Microbiol* (2008) 16(3):107–14. doi: 10.1016/j.tim.2007.12.008
16. Chinen T, Rudensky AY. The effects of commensal microbiota on immune cell subsets and inflammatory responses. *Immunol Rev* (2012) 245(1):45–55. doi: 10.1111/j.1600-065X.2011.01083.x
17. Chervonsky AV. Microbiota and autoimmunity. *Cold Spring Harb Perspect Biol* (2013) 5(3):a007294. doi: 10.1101/cshperspect.a007294
18. Vatanen T, Kostic AD, d'Hennezel E, Siljander H, Franzosa EA, Yassour M, et al. Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. *Cell* (2016) 165(6):1551. doi: 10.1016/j.cell.2016.05.056
19. Davis-Richardson AG, Ardissone AN, Dias R, Simell V, Leonard MT, Kemppainen KM, et al. Bacteroides dorei dominates gut microbiome prior to autoimmunity in Finnish children at high risk for type 1 diabetes. *Front Microbiol* (2014) 5:678. doi: 10.3389/fmicb.2014.00678
20. Lee YK, Menezes JS, Umesaki Y, Mazmanian SK. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* (2011) 108 Suppl 1:4615–22. doi: 10.1073/pnas.1000082107
21. Horai R, Zarate-Blades CR, Dillenburg-Pilla P, Chen J, Kielczewski JL, Silver PB, et al. Microbiota-Dependent Activation of an Autoreactive T Cell Receptor Provokes Autoimmunity in an Immunologically Privileged Site. *Immunity* (2015) 43(2):343–53. doi: 10.1016/j.immuni.2015.07.014
22. Felix KM, Tahsin S, Wu HJ. Host-microbiota interplay in mediating immune disorders. *Ann N Y Acad Sci* (2018) 1417(1):57–70. doi: 10.1111/nyas.13508
23. van der Meulen TA, Harmsen H, Bootsma H, Spijkervet F, Kroese F, Vissink A. The microbiome-systemic diseases connection. *Oral Dis* (2016) 22(8):719–34. doi: 10.1111/odi.12472
24. Ivanov II, Honda K. Intestinal commensal microbes as immune modulators. *Cell Host Microbe* (2012) 12(4):496–508. doi: 10.1016/j.chom.2012.09.009

25. El-Awady AR, Arce RM, Cutler CW. Dendritic cells: microbial clearance via autophagy and potential immunobiological consequences for periodontal disease. *Periodontology* (2000) 201569(1):160–80. doi: 10.1111/prd.12096
26. Potgieter M, Bester J, Kell DB, Pretorius E. The dormant blood microbiome in chronic, inflammatory diseases. *FEMS Microbiol Rev* (2015) 39(4):567–91. doi: 10.1093/femsre/fuv013
27. Krishnan K, Chen T, Paster BJ. A practical guide to the oral microbiome and its relation to health and disease. *Oral Dis* (2017) 23(3):276–86. doi: 10.1111/odi.12509
28. Hajishengallis G, Lamont RJ. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol Oral Microbiol* (2012) 27(6):409–19. doi: 10.1111/j.2041-1014.2012.00663.x
29. Hall MW, Singh N, Ng KF, Lam DK, Goldberg MB, Tenenbaum HC, et al. Inter-personal diversity and temporal dynamics of dental, tongue, and salivary microbiota in the healthy oral cavity. *NPJ Biofilms Microbiomes* (2017) 3:2. doi: 10.1038/s41522-016-0011-0
30. Walker MY, Pratap S, Southerland JH, Farmer-Dixon CM, Lakshmyya K, Gangula PR. Role of oral and gut microbiome in nitric oxide-mediated colon motility. *Nitric Oxide Biol Chem* (2018) 73:81–8. doi: 10.1016/j.niox.2017.06.003
31. Rosier BT, Marsh PD, Mira A. Resilience of the Oral Microbiota in Health: Mechanisms That Prevent Dysbiosis. *J Dental Res* (2018) 97(4):371–80. doi: 10.1177/0022034517742139
32. Zheng D, Liwinski T, Elinav E. Interaction between microbiota and immunity in health and disease. *Cell Res* (2020) 30(6):492–506. doi: 10.1038/s41422-020-0332-7
33. Moutsopoulos NM, Moutsopoulos HM. The oral mucosa: A barrier site participating in tissue-specific and systemic immunity. *Oral Dis* (2018) 24(1-2):22–5. doi: 10.1111/odi.12729
34. Rosier BT, De Jager M, Zaura E, Krom BP. Historical and contemporary hypotheses on the development of oral diseases: are we there yet? *Front Cell Infect Microbiol* (2014) 4:92. doi: 10.3389/fcimb.2014.00092
35. Dahlen G, Fejerskov O, Manji F. Current concepts and an alternative perspective on periodontal disease. *BMC Oral Health* (2020) 20(1):235. doi: 10.1186/s12903-020-01221-4
36. Wolf DL, Neiderud AM, Hinckley K, Dahlen G, van de Winkel JG, Papapanou PN. Fcgamma receptor polymorphisms and periodontal status: a prospective follow-up study. *J Clin Periodontol* (2006) 33(10):691–8. doi: 10.1111/j.1600-051X.2006.00973.x
37. Lamont RJ, Hajishengallis G. Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol Med* (2015) 21(3):172–83. doi: 10.1016/j.molmed.2014.11.004
38. Hajishengallis G, Lamont RJ. Dancing with the Stars: How Choreographed Bacterial Interactions Dictate Nososymbiocity and Give Rise to Keystone Pathogens, Accessory Pathogens, and Pathobionts. *Trends Microbiol* (2016) 24(6):477–89. doi: 10.1016/j.tim.2016.02.010
39. Lamont RJ, Koo H, Hajishengallis G. The oral microbiota: dynamic communities and host interactions. *Nat Rev Microbiol* (2018) 16(12):745–59. doi: 10.1038/s41579-018-0089-x
40. Chen B, Sun L, Zhang X. Integration of microbiome and epigenome to decipher the pathogenesis of autoimmune diseases. *J Autoimmun* (2017) 83:31–42. doi: 10.1016/j.jaut.2017.03.009
41. Rohani MG, Parks WC. Matrix remodeling by MMPs during wound repair. *Matrix Biol J Int Soc Matrix Biol* (2015) 44-46:113–21. doi: 10.1016/j.matbio.2015.03.002
42. Yilmaz M, Kasnak G, Polat NG, Topcuoglu NE, Ku LG, Firatli E. Pathogen profile and MMP-3 levels in areas with varied attachment loss in generalized aggressive and chronic periodontitis. *Cent Eur J Immunol* (2019) 44(4):440–6. doi: 10.5114/ceji.2019.92806
43. Opdenakker G, Dillen C, Fiten P, Martens E, Van Aelst I, Van den Steen PE, et al. Remnant epitopes, autoimmunity and glycosylation. *Biochim Biophys Acta* (2006) 1760(4):610–5. doi: 10.1016/j.bbagen.2005.12.014
44. Opdenakker G, Van Damme J. Cytokine-regulated proteases in autoimmune diseases. *Immunol Today* (1994) 15(3):103–7. doi: 10.1016/0167-5699(94)90151-1
45. Descamps FJ, Van den Steen PE, Nelissen I, Van Damme J, Opdenakker G. Remnant epitopes generate autoimmunity: from rheumatoid arthritis and multiple sclerosis to diabetes. *Adv Exp Med Biol* (2003) 535:69–77. doi: 10.1007/978-1-4615-0065-0_5
46. Opdenakker G, Abu El-Asrar A, Van Damme J. Remnant Epitopes Generating Autoimmunity: From Model to Useful Paradigm. *Trends Immunol* (2020) 41(5):367–78. doi: 10.1016/j.it.2020.03.004
47. Potempa J, Mydel P, Koziel J. The case for periodontitis in the pathogenesis of rheumatoid arthritis. *Nat Rev Rheumatol* (2017) 13(10):606–20. doi: 10.1038/nrrheum.2017.132
48. Vitkov L, Hannig M, Minnich B, Herrmann M. Periodontal sources of citrullinated antigens and TLR agonists related to RA. *Autoimmunity* (2018) 51(6):304–9. doi: 10.1080/08916934.2018.1527907
49. Wu CY, Yang HY, Lai JH. Anti-Citrullinated Protein Antibodies in Patients with Rheumatoid Arthritis: Biological Effects and Mechanisms of Immunopathogenesis. *Int J Mol Sci* (2020) 21(11):1–23. doi: 10.3390/ijms21114015
50. Koziel J, Mydel P, Potempa J. The link between periodontal disease and rheumatoid arthritis: an updated review. Curr. *Rheumatol Rep* (2014) 16(3):408. doi: 10.1007/s11926-014-0408-9
51. Bender P, Burgin WB, Sculean A, Eick S. Serum antibody levels against Porphyromonas gingivalis in patients with and without rheumatoid arthritis—a systematic review and meta-analysis. *Clin Oral Investig* (2017) 21(1):33–42. doi: 10.1007/s00784-016-1938-5
52. Nesse W, Westra J, van der Wal JE, Abbas F, Nicholas AP, Vissink A, et al. The periodontium of periodontitis patients contains citrullinated proteins which may play a role in ACPA (anti-citrullinated protein antibody) formation. *J Clin Periodontol* (2012) 39(7):599–607. doi: 10.1111/j.1600-051X.2012.01885.x
53. Harvey GP, Fitzsimmons TR, Dhamarpatni AA, Marchant C, Haynes DR, Bartold PM. Expression of peptidylarginine deiminase-2 and -4, citrullinated proteins and anti-citrullinated protein antibodies in human gingiva. *J Periodontal Res* (2013) 48(2):252–61. doi: 10.1111/jre.12002
54. Bright R, Proudman SM, Rosenstein ED, Bartold PM. Is there a link between carbamylation and citrullination in periodontal disease and rheumatoid arthritis? *Med Hypotheses* (2015) 84(6):570–6. doi: 10.1016/j.mehy.2015.03.006
55. Harbison JE, Roth-Schulze AJ, Giles LC, Tran CD, Ngui KM, Penno MA, et al. Gut microbiome dysbiosis and increased intestinal permeability in children with islet autoimmunity and type 1 diabetes: A prospective cohort study. *Pediatr Diabetes* (2019) 20(5):574–83. doi: 10.1111/pedi.12865
56. Horta-Baas G, Romero-Figueroa MDS, Montiel-Jarquin AJ, Pizano-Zarate ML, Garcia-Mena J, Ramirez-Duran N. Intestinal Dysbiosis and Rheumatoid Arthritis: A Link between Gut Microbiota and the Pathogenesis of Rheumatoid Arthritis. *J Immunol Res* (2017) 2017:4835189. doi: 10.1155/2017/4835189
57. Zhang X, Zhang D, Jia H, Feng Q, Wang D, Liang D, et al. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. *Nat Med* (2015) 21(8):895–905. doi: 10.1038/nm.3914
58. Bennike TB, Ellingsen T, Glerup H, Bonderup OK, Carlsen TG, Meyer MK, et al. Proteome Analysis of Rheumatoid Arthritis Gut Mucosa. *J Proteome Res* (2017) 16(1):346–54. doi: 10.1021/acs.jproteome.6b00598
59. Liu X, Zeng B, Zhang J, Li W, Mou F, Wang H, et al. Role of the Gut Microbiome in Modulating Arthritis Progression in Mice. *Sci Rep* (2016) 6:30594. doi: 10.1038/srep30594
60. Luckey D, Gomez A, Murray J, White B, Taneja V. Bugs & us: the role of the gut in autoimmunity. *Indian J Med Res* (2013) 138(5):732–43.
61. Negi S, Singh H, Mukhopadhyay A. Gut bacterial peptides with autoimmunity potential as environmental trigger for late onset complex diseases: In-silico study. *PloS One* (2017) 12(7):e0180518. doi: 10.1371/journal.pone.0180518
62. Lerner A, Matthias T. Changes in intestinal tight junction permeability associated with industrial food additives explain the rising incidence of autoimmune disease. *Autoimmun Rev* (2015) 14(6):479–89. doi: 10.1016/j.autrev.2015.01.009
63. Li Q, Lin X, Wu Z, He L, Wang W, Cao Q, et al. Immuno-histochemistry analysis of Helicobacter pylori antigen in renal biopsy specimens from patients with glomerulonephritis. *Saudi J Kidney Dis Transpl* (2013) 24(4):751–8. doi: 10.4103/1319-2442.113871
64. Ko GH, Park HB, Shin MK, Park CK, Lee JH, Youn HS, et al. Monoclonal antibodies against Helicobacter pylori cross-react with human tissue. *Helicobacter* (1997) 2(4):210–5. doi: 10.1111/j.1523-5378.1997.tb00090.x

65. Kato T, Yamazaki K, Nakajima M, Date Y, Kikuchi J, Hase K, et al. Oral Administration of Porphyromonas gingivalis Alters the Gut Microbiome and Serum Metabolome. *mSphere* (2018) 3(5):1–11. doi: 10.1128/mSphere.00460-18
66. Rafferty B, Jonsson D, Kalachikov S, Demmer RT, Nowygrod R, Elkind MS, et al. Impact of monocytic cells on recovery of uncultivable bacteria from atherosclerotic lesions. *J Internal Med* (2011) 270(3):273–80. doi: 10.1111/j.1365-2796.2011.02373.x
67. Vidal F, Figueredo CM, Cordovil I, Fischer RG. Periodontal therapy reduces plasma levels of interleukin-6, C-reactive protein, and fibrinogen in patients with severe periodontitis and refractory arterial hypertension. *J Periodontol* (2009) 80(5):786–91. doi: 10.1902/jop.2009.080471
68. Nakajima M, Arimatsu K, Kato T, Matsuda Y, Minagawa T, Takahashi N, et al. Oral Administration of P. gingivalis Induces Dysbiosis of Gut Microbiota and Impaired Barrier Function Leading to Dissemination of Enterobacteria to the Liver. *PloS One* (2015) 10(7):e0134234. doi: 10.1371/journal.pone.0134234
69. Ohtsu A, Takeuchi Y, Katagiri S, Suda W, Maekawa S, Shiba T, et al. Influence of Porphyromonas gingivalis in gut microbiota of streptozotocin-induced diabetic mice. *Oral Dis* (2019) 25(3):868–80. doi: 10.1111/odi.13044
70. Oldstone MB. Molecular mimicry and autoimmune disease. *Cell* (1987) 50 (6):819–20. doi: 10.1016/0092-8674(87)90507-1
71. Wucherpfennig KW, Strominger JL. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* (1995) 80(5):695–705. doi: 10.1016/0092-8674(95)90348-8
72. Rojas M, Restrepo-Jimenez P, Monsalve DM, Pacheco Y, Acosta-Ampudia Y, Ramirez-Santana C, et al. Molecular mimicry and autoimmunity. *J Autoimmun* (2018) 95:100–23. doi: 10.1016/j.jaut.2018.10.012
73. Albert LJ, Inman RD. Molecular mimicry and autoimmunity. *N Engl J Med* (1999) 341(27):2068–74. doi: 10.1056/NEJM199912303412707
74. Neidel S, Ren H, Torres AA, Smith GL. NF-kappaB activation is a turn on for vaccinia virus phosphoprotein A49 to turn off NF-kappaB activation. *Proc Natl Acad Sci USA* (2019) 116(12):5699–704. doi: 10.1073/pnas.1813504116
75. Brown AS. The environment and susceptibility to schizophrenia. *Prog Neurobiol* (2011) 93(1):23–58. doi: 10.1016/j.pneurobio.2010.09.003
76. Scher JU, Sczesnak A, Longman RS, Segata N, Ubeda C, Bielski C, et al. Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. *Elife* (2013) 2:e01202. doi: 10.7554/eLife.01202
77. Yurkovetskiy LA, Pickard JM, Chervonsky AV. Microbiota and autoimmunity: exploring new avenues. *Cell Host Microbe* (2015) 17(5):548–52. doi: 10.1016/j.chom.2015.04.010
78. Bachmaier K, Neu N, de la Maza LM, Pal S, Hessel A, Penninger JM. Chlamydia infections and heart disease linked through antigenic mimicry. *Science* (1999) 283(5406):1335–9. doi: 10.1126/science.283.5406.1335
79. Martin R, Gran B, Zhao Y, Markovic-Plese S, Bielekova B, Marques A, et al. Molecular mimicry and antigen-specific T cell responses in multiple sclerosis and chronic CNS Lyme disease. *J Autoimmun* (2001) 16(3):187–92. doi: 10.1006/jaut.2000.0501
80. Kaufman DL, Erlander MG, Clare-Salzler M, Atkinson MA, Maclaren NK, Tobin AJ. Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J Clin Invest* (1992) 89(1):283–92. doi: 10.1172/JCI115573
81. Cunningham MW, Krisher K, Graves DC. Murine monoclonal antibodies reactive with human heart and group A streptococcal membrane antigens. *Infect Immun* (1984) 46(1):34–41. doi: 10.1128/IAI.46.1.34-41.1984
82. Wildner G, Diedrichs-Mohring M. Autoimmune uveitis induced by molecular mimicry of peptides from rotavirus, bovine casein and retinal S-antigen. *Eur J Immunol* (2003) 33(9):2577–87. doi: 10.1002/eji.200324058
83. Zhang L, Zhang YJ, Chen J, Huang XL, Fang GS, Yang LJ, et al. The association of HLA-B27 and Klebsiella pneumoniae in ankylosing spondylitis: A systematic review. *Microb Pathog* (2018) 117:49–54. doi: 10.1016/j.micpath.2018.02.020
84. Igoe A, Scofield RH. Autoimmunity and infection in Sjogren's syndrome. *Curr Opin Rheumatol* (2013) 25(4):480–7. doi: 10.1097/BOR.0b013e32836200d2
85. Yamazaki K, Ohsawa Y, Itoh H, Ueki K, Tabeta K, Oda T, et al. T-cell clonality to Porphyromonas gingivalis and human heat shock protein 60s in patients with atherosclerosis and periodontitis. *Oral Microbiol Immunol* (2004) 19(3):160–7. doi: 10.1111/j.0902-0055.2004.00134.x
86. Hinode D, Grenier D, Mayrand D. A general procedure for the isolation of heat-shock proteins from periodontopathogenic bacteria. *J Microbiol Methods* (1996) 25(3):349–55. doi: 10.1016/0167-7012(96)00008-5
87. Tabeta K, Yamazaki K, Hotokezaka H, Yoshie H, Hara K. Elevated humoral immune response to heat shock protein 60 (hsp60) family in periodontitis patients. *Clin Exp Immunol* (2000) 120(2):285–93. doi: 10.1046/j.1365-2249.2000.01216.x
88. Maeda H, Miyamoto M, Kokeguchi S, Kono T, Nishimura F, Takashiba S, et al. Epitope mapping of heat shock protein 60 (GroEL) from Porphyromonas gingivalis. *FEMS Immunol Med Microbiol* (2000) 28 (3):219–24. doi: 10.1111/j.1574-695X.2000.tb01480.x
89. Yamazaki K, Ohsawa Y, Tabeta K, Ito H, Ueki K, Oda T, et al. Accumulation of human heat shock protein 60-reactive T cells in the gingival tissues of periodontitis patients. *Infect Immun* (2002) 70(5):2492–501. doi: 10.1128/IAI.70.5.2492-2501.2002
90. Alfakry H, Paju S, Sinisalo J, Nieminen MS, Valtonen V, Saikku P, et al. Periodontopathogen- and host-derived immune response in acute coronary syndrome. *Scand J Immunol* (2011) 74(4):383–9. doi: 10.1111/j.1365-3083.2011.02584.x
91. Choi J, Lee SY, Kim K, Choi BK. Identification of immunoreactive epitopes of the Porphyromonas gingivalis heat shock protein in periodontitis and atherosclerosis. *J Periodontal Res* (2011) 46(2):240–5. doi: 10.1111/j.1600-0765.2010.01339.x
92. Yamazaki K, Ueki-Maruayama K, Honda T, Nakajima T, Seymour GJ. Effect of periodontal treatment on the serum antibody levels to heat shock proteins. *Clin Exp Immunol* (2004) 135(3):478–82. doi: 10.1111/j.1365-2249.2003.02375.x
93. Schenkein HA, Berry CR, Burmeister JA, Brooks CN, Barbour SE, Best AM, et al. Anti-cardiolipin antibodies in sera from patients with periodontitis. *J Dental Res* (2003) 82(11):919–22. doi: 10.1177/154405910308201114
94. Wang D, Nagasawa T, Chen Y, Ushida Y, Kobayashi H, Takeuchi Y, et al. Molecular mimicry of Aggregatibacter actinomycetemcomitans with beta2 glycoprotein I. *Oral Microbiol Immunol* (2008) 23(5):401–5. doi: 10.1111/j.1399-302X.2008.00442.x
95. Chen YW, Nagasawa T, Wara-Aswapati N, Ushida Y, Wang D, Takeuchi Y, et al. Association between periodontitis and anti-cardiolipin antibodies in Buerger disease. *J Clin Periodontol* (2009) 36(10):830–5. doi: 10.1111/j.1600-051X.2009.01467.x
96. Schenkein HA, Thomas RR. Anticardiolipin (aCL) in sera from periodontitis subjects activate Toll-like receptor 4 (TLR4). *PloS One* (2018) 13(9): e0203494. doi: 10.1371/journal.pone.0203494
97. Leishman SJ, Ford PJ, Do HL, Palmer JE, Heng NC, West MJ, et al. Periodontal pathogen load and increased antibody response to heat shock protein 60 in patients with cardiovascular disease. *J Clin Periodontol* (2012) 39(10):923–30. doi: 10.1111/j.1600-051X.2012.01934.x
98. Buhlin K, Holmer J, Gustafsson A, Horkko S, Pockley AG, Johansson A, et al. Association of periodontitis with persistent, pro-atherogenic antibody responses. *J Clin Periodontol* (2015) 42(11):1006–14. doi: 10.1111/jcpe.12456
99. Mikolajczyk TP, Guzik TJ. Adaptive Immunity in Hypertension. *Curr Hypertens Rep* (2019) 21(9):68. doi: 10.1007/s11906-019-0971-6
100. Pacheco Y, Acosta-Ampudia Y, Monsalve DM, Chang C, Gershwin ME, Anaya JM. Bystander activation and autoimmunity. *J Autoimmun* (2019) 103:102301. doi: 10.1016/j.jaut.2019.06.012
101. Garg A, Kumari B, Singhal N, Kumar M. Using molecular-mimicry-inducing pathways of pathogens as novel drug targets. *Drug Discov Today* (2019) 24 (9):1943–52. doi: 10.1016/j.drudis.2018.10.010
102. Scherer MT, Ignatowicz L, Winslow GM, Kappler JW, Marrack P. Superantigens: bacterial and viral proteins that manipulate the immune system. *Annu Rev Cell Biol* (1993) 9:101–28. doi: 10.1146/annurev.cb.09.110193.000533
103. Tlaskalova-Hogenova H, Stepankova R, Hudcovic T, Tuckova L, Cukrowska B, Lodinova-Zadnikova R, et al. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. *Immunol Lett* (2004) 93(2-3):97–108. doi: 10.1016/j.imlet.2004.02.005
104. Rink L, Luhm J, Koester M, Kirchner H. Induction of a cytokine network by superantigens with parallel TH1 and TH2 stimulation. *J Interferon Cytokine Res* (1996) 16(1):41–7. doi: 10.1089/jir.1996.16.41
105. Tomar N, De RK. A model of an integrated immune system pathway in Homo sapiens and its interaction with superantigen producing expression regulatory pathway in Staphylococcus aureus: comparing behavior of pathogen perturbed and unperturbed pathway. *PloS One* (2013) 8(12): e80918. doi: 10.1371/journal.pone.0080918

106. Nakajima T, Yamazaki K, Hara K. Biased T cell receptor V gene usage in tissues with periodontal disease. *J Periodontal Res* (1996) 31(1):2–10. doi: 10.1111/j.1600-0765.1996.tb00457.x
107. Zadeh HH, Kreutzer DL. Evidence for involvement of superantigens in human periodontal diseases: skewed expression of T cell receptor variable regions by gingival T cells. *Oral Microbiol Immunol* (1996) 11(2):88–95. doi: 10.1111/j.1399-302X.1996.tb00341.x
108. Mathur A, Michalowicz B, Yang C, Aeppli D. Influence of periodontal bacteria and disease status on V beta expression in T cells. *J Periodontal Res* (1995) 30(5):369–73. doi: 10.1111/j.1600-0765.1995.tb01289.x
109. Leung KP, Torres BA. Prevotella intermedia stimulates expansion of Vbeta-specific CD4(+) T cells. *Infect Immun* (2000) 68(9):5420–4. doi: 10.1128/IAI.68.9.5420-5424.2000
110. Petit MD, Stashenko P. Extracts of periodontopathic microorganisms lack functional superantigenic activity for murine T cells. *J Periodontal Res* (1996) 31(8):517–24. doi: 10.1111/j.1600-0765.1996.tb00515.x
111. Gemmell E, Grieco DA, Cullinan MP, Westerman B, Seymour GJ. Antigen-specific T-cell receptor V beta expression in Porphyromonas gingivalis-specific T-cell lines. *Oral Microbiol Immunol* (1998) 13(6):355–61. doi: 10.1111/j.1399-302X.1998.tb00691.x
112. Berglundh T, Donati M. Aspects of adaptive host response in periodontitis. *J Clin Periodontol* (2005) 32 Suppl 6:87–107. doi: 10.1111/j.1600-051X.2005.00820.x
113. Burnham JP, Kollef MH. Understanding toxic shock syndrome. *Intensive Care Med* (2015) 41(9):1707–10. doi: 10.1007/s00134-015-3861-7
114. Muluk NB, Altin F, Cingi C. Role of Superantigens in Allergic Inflammation: Their Relationship to Allergic Rhinitis, Chronic Rhinosinusitis, Asthma, and Atopic Dermatitis. *Am J Rhinol Allergy* (2018) 32(6):502–17. doi: 10.1177/1945892418801083
115. Spaulding AR, Salgado-Pabon W, Kohler PL, Horswill AR, Leung DY, Schlievert PM. Staphylococcal and streptococcal superantigen exotoxins. *Clin Microbiol Rev* (2013) 26(3):422–47. doi: 10.1128/CMR.00104-12
116. Radcliff FJ, Waldvogel-Thurlow S, Clow F, Mahadevan M, Johnston J, Li G, et al. Impact of Superantigen-Producing Bacteria on T Cells from Tonsillar Hyperplasia. *Pathogens* (2019) 8(3):1–17. doi: 10.3390/pathogens8030090
117. Andrews LP, Yano H, Vignali DAA. Inhibitory receptors and ligands beyond PD-1, PD-L1 and CTLA-4: breakthroughs or backups. *Nat Immunol* (2019) 20(11):1425–34. doi: 10.1038/s41590-019-0512-0
118. Duraiswamy J, Freeman GJ, Coukos G. Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors–response. *Cancer Res* (2014) 74(2):633–4; discussion 5. doi: 10.1158/0008-5472.CAN-13-2752
119. Butt AQ, Mills KH. Immunosuppressive networks and checkpoints controlling antitumor immunity and their blockade in the development of cancer immunotherapeutics and vaccines. *Oncogene* (2014) 33(38):4623–31. doi: 10.1038/onc.2013.432
120. Ha SJ, Mueller SN, Wherry EJ, Barber DL, Aubert RD, Sharpe AH, et al. Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. *J Exp Med* (2008) 205(3):543–55. doi: 10.1084/jem.20071949
121. Dyck L, Mills KHG. Immune checkpoints and their inhibition in cancer and infectious diseases. *Eur J Immunol* (2017) 47(5):765–79. doi: 10.1002/eji.201646875
122. Paluch C, Santos AM, Anzilotti C, Cornall RJ, Davis SJ. Immune Checkpoints as Therapeutic Targets in Autoimmunity. *Front Immunol* (2018) 9:2306. doi: 10.3389/fimmu.2018.02306
123. Houshmand B, Rafiei A, Hajilooi M. Influence of cytotoxic T lymphocyte antigen-4 (CTLA-4) gene polymorphisms in periodontitis. *Arch Oral Biol* (2012) 57(9):1218–24. doi: 10.1016/j.archoralbio.2012.03.002
124. e Silva MR, Moreira PR, da Costa GC, Saraiva AM, de Souza PE, Amormino SA, et al. Association of CD28 and CTLA-4 gene polymorphisms with aggressive periodontitis in Brazilians. *Oral Dis* (2013) 19(6):568–76. doi: 10.1111/odi.12036
125. He F, Zhou Y, Wang X, Li L, Geng Y, Wang Z, et al. Functional Polymorphisms of CTLA4 Associated with Aggressive Periodontitis in the Chinese Han Population. *Cell Physiol Biochem* (2018) 50(3):1178–85. doi: 10.1159/000494544
126. Zamani MR, Aslani S, Salmaninejad A, Javan MR, Rezaei N. PD-1/PD-L and autoimmunity: A growing relationship. *Cell Immunol* (2016) 310:27–41. doi: 10.1016/j.cellimm.2016.09.009
127. Olsen I, Taubman MA, Singhrao SK. Porphyromonas gingivalis suppresses adaptive immunity in periodontitis, atherosclerosis, and Alzheimer's disease. *J Oral Microbiol* (2016) 8:33029. doi: 10.3402/jom.v8.33029
128. Bailly C. The implication of the PD-1/PD-L1 checkpoint in chronic periodontitis suggests novel therapeutic opportunities with natural products. *Jpn Dent Sci Rev* (2020) 56(1):90–6. doi: 10.1016/j.jdsr.2020.04.002
129. Gaddis DE, Maynard CL, Weaver CT, Michalek SM, Katz J. Role of TLR2-dependent IL-10 production in the inhibition of the initial IFN-gamma T cell response to Porphyromonas gingivalis. *J Leukoc Biol* (2013) 93(1):21–31. doi: 10.1189/jlb.0512220
130. Holmgren AM, McConkey CA, Shin S. Outrunning the Red Queen: bystander activation as a means of outpacing innate immune subversion by intracellular pathogens. *Cell Mol Immunol* (2017) 14(1):14–21. doi: 10.1038/cmi.2016.36
131. Seyda M, Elkhal A, Quante M, Falk CS, Tullius SG. T Cells Going Innate. *Trends Immunol* (2016) 37(8):546–56. doi: 10.1016/j.it.2016.06.004
132. Reynolds JM, Dong C. Toll-like receptor regulation of effector T lymphocyte function. *Trends Immunol* (2013) 34(10):511–9. doi: 10.1016/j.it.2013.06.003
133. Tough DF, Borrow P, Sprent J. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science* (1996) 272(5270):1947–50. doi: 10.1126/science.272.5270.1947
134. Kamath AT, Sheasby CE, Tough DF. Dendritic cells and NK cells stimulate bystander T cell activation in response to TLR agonists through secretion of IFN-alpha beta and IFN-gamma. *J Immunol* (2005) 174(2):767–76. doi: 10.4049/jimmunol.174.2.767
135. Whiteside SK, Snook JP, Williams MA, Weis JJ. Bystander T Cells: A Balancing Act of Friends and Foes. *Trends Immunol* (2018) 39(12):1021–35. doi: 10.1016/j.it.2018.10.003
136. Van Kaer L. Innate and virtual memory T cells in man. *Eur J Immunol* (2015) 45(7):1916–20. doi: 10.1002/eji.201545761
137. Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, et al. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* (2007) 449(7162):564–9. doi: 10.1038/nature06116
138. Ganguly D, Chamilos G, Lande R, Gregorio J, Meller S, Facchinetti V, et al. Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. *J Exp Med* (2009) 206(9):1983–94. doi: 10.1084/jem.20090480
139. Majer O, Liu B, Barton GM. Nucleic acid-sensing TLRs: trafficking and regulation. *Curr Opin Immunol* (2017) 44:26–33. doi: 10.1016/j.coi.2016.10.003
140. Wong FS, Hu C, Zhang L, Du W, Alexopoulou L, Flavell RA, et al. The role of Toll-like receptors 3 and 9 in the development of autoimmune diabetes in NOD mice. *Ann N Y Acad Sci* (2008) 1150:146–8. doi: 10.1196/annals.1447.039
141. Burrows MP, Volchkov P, Kobayashi KS, Chervonsky AV. Microbiota regulates type 1 diabetes through Toll-like receptors. *Proc Natl Acad Sci USA* (2015) 112(32):9973–7. doi: 10.1073/pnas.1508740112
142. Jin L, Ren L, Leung WK, Darveau RP. The in vivo expression of membrane-bound CD14 in periodontal health and disease. *J Periodontol* (2004) 75(4):578–85. doi: 10.1902/jop.2004.75.4.578
143. Beklen A, Hukkanen M, Richardson R, Konttinen YT. Immunohistochemical localization of Toll-like receptors 1-10 in periodontitis. *Oral Microbiol Immunol* (2008) 23(5):425–31. doi: 10.1111/j.1399-302X.2008.00448.x
144. Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol* (2005) 5(6):446–58. doi: 10.1038/nri1630
145. Isaza-Guzman DM, Aristizabal-Cardona D, Martinez-Pabon MC, Velasquez-Echeverri H, Tobon-Arroyave SI. Estimation of sCD14 levels in saliva obtained from patients with various periodontal conditions. *Oral Dis* (2008) 14(5):450–6. doi: 10.1111/j.1601-0825.2007.01400.x
146. AlQallaf H, Hamada Y, Blanchard S, Shin D, Gregory R, Srinivasan M. Differential profiles of soluble and cellular toll like receptor (TLR)-2 and 4 in chronic periodontitis. *PloS One* (2018) 13(12):e0200231. doi: 10.1371/journal.pone.0200231
147. Muthukuru M, Jotwani R, Cutler CW. Oral mucosal endotoxin tolerance induction in chronic periodontitis. *Infect Immun* (2005) 73(21):687–94. doi: 10.1128/IAI.73.2.687-694.2005
148. Wu X, Zhang G, Wang X, Zhang P, Tan Y. Endotoxin tolerance induction in human periodontal ligament fibroblasts stimulated with different bacterial

lipopolysaccharides. *Arch Oral Biol* (2015) 60(3):463–70. doi: 10.1016/j.archoralbio.2014.10.005
149. Wei W, Ren J, Yin W, Ding H, Lu Q, Tan L, et al. Inhibition of Ctsk modulates periodontitis with arthritis via downregulation of TLR9 and autophagy. *Cell Prolif* (2020) 53(1):e12722. doi: 10.1111/cpr.12722
150. Song B, Zhang YL, Chen LJ, Zhou T, Huang WK, Zhou X, et al. The role of Toll-like receptors in periodontitis. *Oral Dis* (2017) 23(2):168–80. doi: 10.1111/odi.12468
151. Tsai IS, Tsai CC, Ho YP, Ho KY, Wu YM, Hung CC. Interleukin-12 and interleukin-16 in periodontal disease. *Cytokine* (2005) 31(1):34–40. doi: 10.1016/j.cyto.2005.02.007
152. Yucel OO, Berker E, Gariboglu S, Otlu H. Interleukin-11, interleukin-1beta, interleukin-12 and the pathogenesis of inflammatory periodontal diseases. *J Clin Periodontol* (2008) 35(5):365–70. doi: 10.1111/j.1600-051X.2008.01212.x
153. Honda T, Aoki Y, Takahashi N, Maekawa T, Nakajima T, Ito H, et al. Elevated expression of IL-17 and IL-12 genes in chronic inflammatory periodontal disease. *Clin Chim Acta Int J Clin Chem* (2008) 395(1-2):137–41. doi: 10.1016/j.cca.2008.06.003
154. Reichert S, Machulla HK, Klapproth J, Zimmermann U, Reichert Y, Glaser C, et al. Interferon-gamma and interleukin-12 gene polymorphisms and their relation to aggressive and chronic periodontitis and key periodontal pathogens. *J Periodontol* (2008) 79(8):1434–43. doi: 10.1902/jop.2008.070637
155. Sasaki H, Suzuki N, Kent RJr., Kawashima N, Takeda J, Stashenko P. T cell response mediated by myeloid cell-derived IL-12 is responsible for Porphyromonas gingivalis-induced periodontitis in IL-10-deficient mice. *J Immunol* (2008) 180(9):6193–8. doi: 10.4049/jimmunol.180.9.6193
156. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* (2006) 24(2):386–98. doi: 10.1634/stemcells.2005-0008
157. Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* (2004) 103(12):4619–21. doi: 10.1182/blood-2003-11-3909
158. Issaranggun Na Ayuthaya B, Everts V, Pavasant P. The immunopathogenic and immunomodulatory effects of interleukin-12 in periodontal disease. *Eur J Oral Sci* (2018) 126(2):75–83. doi: 10.1111/eos.12405
159. Campisi L, Barbet G, Ding Y, Esplugues E, Flavell RA, Blander JM. Apoptosis in response to microbial infection induces autoreactive TH17 cells. *Nat Immunol* (2016) 17(9):1084–92. doi: 10.1038/ni.3512
160. Souza DG, Vieira AT, Soares AC, Pinho V, Nicoli JR, Vieira LQ, et al. The essential role of the intestinal microbiota in facilitating acute inflammatory responses. *J Immunol* (2004) 173(6):4137–46. doi: 10.4049/jimmunol.173.6.4137
161. Khader SA, Gaffen SL, Kolls JK. Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa. *Mucosal Immunol* (2009) Sep2(5):403–11. doi: 10.1038/mi.2009.100
162. Zenobia C, Hajishengallis G. Basic biology and role of interleukin-17 in immunity and inflammation. *Periodontology* (2000) 201569(1):142–59. doi: 10.1111/prd.12083
163. Lubberts E. IL-17/Th17 targeting: on the road to prevent chronic destructive arthritis? *Cytokine* (2008) 41(2):84–91. doi: 10.1016/j.cyto.2007.09.014
164. Beklen A, Ainola M, Hukkanen M, Gurgan C, Sorsa T, Konttinen YT. MMPs, IL-1, and TNF are regulated by IL-17 in periodontitis. *J Dental Res* (2007) 86(4):347–51. doi: 10.1177/154405910708600409
165. Hajishengallis G, Lamont RJ. Breaking bad: manipulation of the host response by Porphyromonas gingivalis. *Eur J Immunol* (2014) 44(2):328–38. doi: 10.1002/eji.201344202
166. Knochelmann HM, Dwyer CJ, Bailey SR, Amaya SM, Elston DM, Mazza-McCrann JM, et al. When worlds collide: Th17 and Treg cells in cancer and autoimmunity. *Cell Mol Immunol* (2018) 15(5):458–69. doi: 10.1038/s41423-018-0004-4
167. Nistala K, Adams S, Cambrook H, Ursu S, Olivito B, de Jager W, et al. Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment. *Proc Natl Acad Sci U S A* (2010) 107(33):14751–6. doi: 10.1073/pnas.1003852107
168. Bsat M, Chapuy L, Rubio M, Wassef R, Richard C, Schwenter F, et al. Differential Pathogenic Th17 Profile in Mesenteric Lymph Nodes of Crohn's Disease and Ulcerative Colitis Patients. *Front Immunol* (2019) 10:1177. doi: 10.3389/fimmu.2019.01177
169. Wade SM, Canavan M, McGarry T, Low C, Wade SC, Mullan RH, et al. Association of synovial tissue polyfunctional T-cells with DAPSA in psoriatic arthritis. *Ann Rheum Dis* (2019) 78(3):350–4. doi: 10.1136/annrheumdis-2018-214138
170. Bittner-Eddy PD, Fischer LA, Costalonga M. Transient Expression of IL-17A in Foxp3 Fate-Tracked Cells in Porphyromonas gingivalis-Mediated Oral Dysbiosis. *Front Immunol* (2020) 11:677. doi: 10.3389/fimmu.2020.00677
171. Dutzan N, Kajikawa T, Abusleme L, Greenwell-Wild T, Zuazo CE, Ikeuchi T, et al. A dysbiotic microbiome triggers TH17 cells to mediate oral mucosal immunopathology in mice and humans. *Sci Transl Med* (2018) 10(463) 1–27. doi: 10.1126/scitranslmed.aat0797
172. Cornaby C, Gibbons L, Mayhew V, Sloan CS, Welling A, Poole BD. B cell epitope spreading: mechanisms and contribution to autoimmune diseases. *Immunol Lett* (2015) 163(1):56–68. doi: 10.1016/j.imlet.2014.11.001
173. Kurien BT, Scofield RH. Autoimmunity and oxidatively modified autoantigens. *Autoimmun Rev* (2008) 7(7):567–73. doi: 10.1016/j.autrev.2008.04.019
174. McCluskey J, Farris AD, Keech CL, Purcell AW, Rischmueller M, Kinoshita G, et al. Determinant spreading: lessons from animal models and human disease. *Immunol Rev* (1998) 164:209–29. doi: 10.1111/j.1600-065X.1998.tb01222.x
175. Kwon EY, Cha GS, Jeong E, Lee JY, Kim SJ, Surh CD, et al. Pep19 drives epitope spreading in periodontitis and periodontitis-associated autoimmune diseases. *J Periodontal Res* (2016) 51(3):381–94. doi: 10.1111/jre.12318
176. Jeong E, Lee JY, Kim SJ, Choi J. Predominant immunoreactivity of Porphyromonas gingivalis heat shock protein in autoimmune diseases. *J Periodontal Res* (2012) 47(6):811–6. doi: 10.1111/j.1600-0765.2012.01501.x
177. Kim HJ, Cha GS, Joo JY, Lee J, Kim SJ, Lee J, et al. Targeting the epitope spreader Pep19 by naive human CD45RA(+) regulatory T cells dictates a distinct suppressive T cell fate in a novel form of immunotherapy. *J Periodontal Implant Sci* (2017) 47(5):292–311. doi: 10.5051/jpis.2017.47.5.292
178. Pendergraft WF 3rd, Badhwar AK, Preston GA. Autoantigen complementarity and its contributions to hallmarks of autoimmune disease. *J Theor Biol* (2015) 375:88–94. doi: 10.1016/j.jtbi.2014.12.006
179. Pendergraft WF 3rd, Preston GA, Shah RR, Tropsha A, Carter CW Jr, JC J, et al. Autoimmunity is triggered by cPR-3(105-201), a protein complementary to human autoantigen proteinase-3. *Nat Med* (2004) 10 (1):72–9. doi: 10.1038/nm968
180. Schultz DR, Tozman EC. Antineutrophil cytoplasmic antibodies: major autoantigens, pathophysiology, and disease associations. *Semin Arthritis Rheumatol* (1995) 25(3):143–59. doi: 10.1016/S0049-0172(95)80027-1
181. Falk RJ, Terrell RS, Charles LA, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. *Proc Natl Acad Sci USA* (1990) 87(11):4115–9. doi: 10.1073/pnas.87.11.4115
182. Utz PJ, Anderson P. Posttranslational protein modifications, apoptosis, and the bypass of tolerance to autoantigens. *Arthritis Rheum* (1998) 41(7):1152–60. doi: 10.1002/1529-0131(199807)41:7<1152::AID-ART3>3.0.CO;2-L
183. Sharma CG, Pradeep AR. Anti-neutrophil cytoplasmic autoantibodies: a renewed paradigm in periodontal disease pathogenesis? *J Periodontol* (2006) 77(8):1304–13. doi: 10.1902/jop.2006.050308
184. Tacnet-Delorme P, Gabillet J, Chatfield S, Thieblemont N, Frachet P, Witko-Sarsat V. Proteinase 3 Interferes With C1q-Mediated Clearance of Apoptotic Cells. *Front Immunol* (2018) 9:818. doi: 10.3389/fimmu.2018.00818
185. Mikuls TR, Payne JB, Reinhardt RA, Thiele GM, Maziarz E, Cannella AC, et al. Antibody responses to Porphyromonas gingivalis (P. gingivalis) in subjects with rheumatoid arthritis and periodontitis. *Int Immunopharmacol* (2009) 9(1):38–42. doi: 10.1016/j.intimp.2008.09.008
186. Liao F, Li Z, Wang Y, Shi B, Gong Z, Cheng X. Porphyromonas gingivalis may play an important role in the pathogenesis of periodontitis-associated rheumatoid arthritis. *Med Hypotheses* (2009) 72(6):732–5. doi: 10.1016/j.mehy.2008.12.040
187. Berthelot JM, Le Goff B. Rheumatoid arthritis and periodontal disease. *Joint Bone Spine* (2010) 77(6):537–41. doi: 10.1016/j.jbspin.2010.04.015
188. Bozkurt FY, Yetkin Ay Z, Berker E, Tepe E, Akkus S. Anti-inflammatory cytokines in gingival crevicular fluid in patients with periodontitis and

rheumatoid arthritis: a preliminary report. *Cytokine* (2006) 35(3-4):180–5. doi: 10.1016/j.cyto.2006.07.020
189. Manolova I, Dancheva M, Halacheva K. Antineutrophil cytoplasmic antibodies in patients with systemic lupus erythematosus: prevalence, antigen specificity, and clinical associations. *Rheumatol Int* (2001) 20 (5):197–204. doi: 10.1007/s002960100108
190. Spronk PE, Bootsma H, Horst G, Huitema MG, Limburg PC, Tervaert JW, et al. Antineutrophil cytoplasmic antibodies in systemic lupus erythematosus. *Br J Rheumatol* (1996) 35(7):625–31. doi: 10.1093/rheumatology/35.7.625
191. Novo E, Garcia-MacGregor E, Viera N, Chaparro N, Crozzoli Y. Periodontitis and anti-neutrophil cytoplasmic antibodies in systemic lupus erythematosus and rheumatoid arthritis: a comparative study. *J Periodontol* (1999) 70(2):185–8. doi: 10.1902/jop.1999.70.2.185
192. Gamonal J, Sanz M, O'Connor A, Acevedo A, Suarez I, Sanz A, et al. Delayed neutrophil apoptosis in chronic periodontitis patients. *J Clin Periodontol* (2003) 30(7):616–23. doi: 10.1034/j.1600-051X.2003.00350.x
193. Gemmell E, Yamazaki K, Seymour GJ. Destructive periodontitis lesions are determined by the nature of the lymphocytic response. *Crit Rev Oral Biol Med* (2002) 13(1):17–34. doi: 10.1177/154411130201300104
194. Govze Y, Herzberg MC. Serum and gingival crevicular fluid anti-desmosomal antibodies in periodontitis. *J Periodontol* (1993) 64(7):603–8. doi: 10.1902/jop.1993.64.7.603
195. Koutouzis T, Haber D, Shaddox L, Aukhil I, Wallet SM. Autoreactivity of serum immunoglobulin to periodontal tissue components: a pilot study. *J Periodontol* (2009) 80(4):625–33. doi: 10.1902/jop.2009.080422
196. Anusaksathien O, Singh G, Matthews N, Dolby AE. Autoimmunity to collagen in adult periodontal disease: immunoglobulin classes in sera and tissue. *J Periodontal Res* (1992) 27(1):55–61. doi: 10.1111/j.1600-0765.1992.tb02086.x
197. Anusaksathien O, Singh G, Peters TJ, Dolby AE. Immunity to self-antigens in periodontal disease. *J Periodontol* (1992) 63(3):194–9. doi: 10.1902/jop.1992.63.3.194
198. Refaie A, Anuksaksathiem O, Singh G, Moran J, Dolby AE. Antibody to collagen type I in gingival crevicular fluid. *J Periodontol* (1990) 61(5):289–92. doi: 10.1902/jop.1990.61.5.289
199. Rajapakse PS, Dolby AE. Evidence for local production of antibodies to auto and non-self antigens in periodontal disease. *Oral Dis* (2004) 10(2):99–105. doi: 10.1046/j.1354-523X.2003.00961.x
200. Sugawara M, Yamashita K, Yoshie H, Hara K. Detection of, and anti-collagen antibody produced by, CD5-positive B cells in inflamed gingival tissues. *J Periodontal Res* (1992) 27(5):489–98. doi: 10.1111/j.1600-0765.1992.tb01822.x
201. Hirsch HZ, Tarkowski A, Miller EJ, Gay S, Koopman WJ, Mestecky J. Autoimmunity to collagen in adult periodontal disease. *J Oral Pathol* (1988) 17(9-10):456–9. doi: 10.1111/j.1600-0714.1988.tb01315.x
202. Ye P, Simonian M, Nadkarni MA, Decarlo AA, Chapple CC, Hunter N. Identification of epithelial auto-antigens associated with periodontal disease. *Clin Exp Immunol* (2005) 139(2):328–37. doi: 10.1111/j.1365-2249.2005.02692.x
203. Conigliaro P, Triggianese P, Ballanti E, Perricone C, Perricone R, Chimenti MS. Complement, infection, and autoimmunity. *Curr Opin Rheumatol* (2019) 31(5):532–41. doi: 10.1097/BOR.0000000000000633
204. Shields KJ, Mollnes TE, Eidet JR, Mikkelsen K, Almdahl SM, Bottazzi B, et al. Plasma complement and vascular complement deposition in patients with coronary artery disease with and without inflammatory rheumatic diseases. *PloS One* (2017) 12(3):e0174577. doi: 10.1371/journal.pone.0174577
205. Herrada AA, Escobedo N, Iruretagoyena M, Valenzuela RA, Burgos PI, Cuitino L, et al. Innate Immune Cells' Contribution to Systemic Lupus Erythematosus. *Front Immunol* (2019) 10:772. doi: 10.3389/fimmu.2019.00772
206. West EE, Kolev M, Kemper C. Complement and the Regulation of T Cell Responses. *Annu Rev Immunol* (2018) 36:309–38. doi: 10.1146/annurev-immunol-042617-053245
207. Markiewski MM, Lambris JD. The role of complement in inflammatory diseases from behind the scenes into the spotlight. *Am J Pathol* (2007) 171 (3):715–27. doi: 10.2353/ajpath.2007.070166
208. Endo M, Ohi H, Ohsawa I, Fujita T, Matsushita M. Complement activation through the lectin pathway in patients with Henoch-Schonlein purpura nephritis. *Am J Kidney Dis* (2000) 35(3):401–7. doi: 10.1016/S0272-6386(00)70192-2
209. Takamori Y, Atsuta I, Nakamura H, Sawase T, Koyano K, Hara Y. Histopathological comparison of the onset of peri-implantitis and periodontitis in rats. *Clin Oral Implants Res* (2017) 28(2):163–70. doi: 10.1111/clr.12777
210. Brajovic G, Stefanovic G, Ilic V, Petrovic S, Stefanovic N, Nikolic-Jakoba N, et al. Association of fibronectin with hypogalactosylated immunoglobulin G in gingival crevicular fluid in periodontitis. *J Periodontol* (2010) 81(10):1472–80. doi: 10.1902/jop.2010.100053
211. Lambris JD, Hajishengallis G. Innate immunity mechanisms. *Adv Exp Med Biol* (2012) 946. doi: 10.1007/978-1-4614-0106-3
212. Damgaard C, Holmstrup P, Van Dyke TE, Nielsen CH. The complement system and its role in the pathogenesis of periodontitis: current concepts. *J Periodontal Res* (2015) 50(3):283–93. doi: 10.1111/jre.12209
213. Larsson L, Castilho RM, Giannobile WV. Epigenetics and its role in periodontal diseases: a state-of-the-art review. *J Periodontol* (2015) 86 (4):556–68. doi: 10.1902/jop.2014.140559
214. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. *Lancet* (2016) 388 (10055):2023–38. doi: 10.1016/S0140-6736(16)30173-8
215. Lei W, Luo Y, Lei W, Luo Y, Yan K, Zhao S, et al. Abnormal DNA methylation in CD4+ T cells from patients with systemic lupus erythematosus, systemic sclerosis, and dermatomyositis. *Scand J Rheumatol* (2009) 38(5):369–74. doi: 10.1080/03009740902758875
216. Yin H, Zhao M, Wu X, Gao F, Luo Y, Ma L, et al. Hypomethylation and overexpression of CD70 (TNFSF7) in CD4+ T cells of patients with primary Sjogren's syndrome. *J Dermatol Sci* (2010) 59(3):198–203. doi: 10.1016/j.jdermsci.2010.06.011
217. Coit P, Yalavarthi S, Ognenovski M, Zhao W, Hasni S, Wren JD, et al. Epigenome profiling reveals significant DNA demethylation of interferon signature genes in lupus neutrophils. *J Autoimmun* (2015) 58:59–66. doi: 10.1016/j.jaut.2015.01.004
218. Richardson B. Primer: epigenetics of autoimmunity. *Nat Clin Pract Rheumatol* (2007) 3(9):521–7. doi: 10.1038/ncprheum0573
219. Hedrich CM, Rauen T, Apostolidis SA, Grammatikos AP, Rodriguez Rodriguez N, Ioannidis C, et al. Stat3 promotes IL-10 expression in lupus T cells through trans-activation and chromatin remodeling. *Proc Natl Acad Sci USA* (2014) 111(37):13457–62. doi: 10.1073/pnas.1408023111
220. Barros SP, Hefni E, Nepomuceno R, Offenbacher S, North K. Targeting epigenetic mechanisms in periodontal diseases. *Periodont 2000* (2018) 78 (1):174–84. doi: 10.1111/prd.12231
221. Martins MD, Jiao Y, Larsson L, Almeida LO, Garaicoa-Pazmino C, Le JM, et al. Epigenetic Modifications of Histones in Periodontal Disease. *J Dental Res* (2016) 95(2):215–22. doi: 10.1177/0022034515611876
222. Brown EM, Kenny DJ, Xavier RJ. Gut Microbiota Regulation of T Cells During Inflammation and Autoimmunity. *Annu Rev Immunol* (2019) 37:599–624. doi: 10.1146/annurev-immunol-042718-041841
223. Elmanfi S, Sintim HO, Zhou J, Gursoy M, Kononen E, Gursoy UK. Activation of Gingival Fibroblasts by Bacterial Cyclic Dinucleotides and Lipopolysaccharide. *Pathogens* (2020) 9(10) 1–14. doi: 10.3390/pathogens9100792
224. Ke X, Lei L, Li H, Li H, Yan F. Manipulation of necroptosis by Porphyromonas gingivalis in periodontitis development. *Mol Immunol* (2016) 77:8–13. doi: 10.1016/j.molimm.2016.07.010
225. Palioto DB, Finoti LS, Kinane DF, Benakanakere M. Epigenetic and inflammatory events in experimental periodontitis following systemic microbial challenge. *J Clin Periodontol* (2019) 46(8):819–29. doi: 10.1111/jcpe.13151
226. Mozzi A, Pontremoli C, Sironi M. Genetic susceptibility to infectious diseases: Current status and future perspectives from genome-wide approaches. *Infect Genet Evol J Mol Epidemiol evolutionary Genet Infect Dis* (2018) 66:286–307. doi: 10.1016/j.meegid.2017.09.028
227. Zhang S, Yu N, Arce RM. Periodontal inflammation: Integrating genes and dysbiosis. *Periodont 2000* (2020) 82(1):129–42. doi: 10.1111/prd.12267
228. Khera AV, Chaffin M, Aragam KG, Haas ME, Roselli C, Choi SH, et al. Genome-wide polygenic scores for common diseases identify individuals with risk equivalent to monogenic mutations. *Nat Genet* (2018) 50(9):1219–24. doi: 10.1038/s41588-018-0183-z
229. Eke PI, Dye BA, Wei L, Slade GD, Thornton-Evans GO, Borgnakke WS, et al. Update on Prevalence of Periodontitis in Adults in the United States: NHANES 2009 to 2012. *J Periodontol* (2015) 86(5):611–22. doi: 10.1902/jop.2015.140520
230. Marcenes W, Kassebaum NJ, Bernabe E, Flaxman A, Naghavi M, Lopez A, et al. Global burden of oral conditions in 1990-2010: a systematic analysis. *J Dental Res* (2013) 92(7):592–7. doi: 10.1177/0022034513490168

231. Lertpimonchai A, Rattanasiri S, Arj-Ong VS, Attia J, Thakkinstian A. The association between oral hygiene and periodontitis: a systematic review and meta-analysis. *Int Dent J* (2017) 67(6):332–43. doi: 10.1111/idj.12317
232. Tribble GD, Lamont RJ. Bacterial invasion of epithelial cells and spreading in periodontal tissue. *Periodont 2000* (2010) 52(1):68–83. doi: 10.1111/j.1600-0757.2009.00323.x
233. Thorbert-Mros S, Larsson L, Kalm J, Berglundh T. Interleukin-17-producing T cells and interleukin-17 mRNA expression in periodontitis and long-standing gingivitis lesions. *J Periodontol* (2019) 90(5):516–21. doi: 10.1002/JPER.18-0326
234. Ebersole JL, Dawson DR,3, Morford LA, Peyyala R, Miller CS, Gonzalez OA. Periodontal disease immunology: 'double indemnity' in protecting the host. *Periodont 2000* (2013) 62(1):163–202. doi: 10.1111/prd.12005
235. Cheng WC, van Asten SD, Burns LA, Evans HG, Walter GJ, Hashim A, et al. Periodontitis-associated pathogens P. gingivalis and A. actinomycetemcomitans activate human CD14(+) monocytes leading to enhanced Th17/IL-17 responses. *Eur J Immunol* (2016) 46(9):2211–21. doi: 10.1002/eji.201545871
236. De Luca F, Shoenfeld Y. The microbiome in autoimmune diseases. *Clin Exp Immunol* (2019) 195(1):74–85. doi: 10.1111/cei.13158

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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Rigorous Plasma Microbiome Analysis Method Enables Disease Association Discovery in Clinic

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Blood microbiome is important to investigate microbial-host interactions and the effects on systemic immune perturbations. However, this effort has met with major challenges due to low microbial biomass and background artifacts. In the current study, microbial 16S DNA sequencing was applied to analyze plasma microbiome. We have developed a quality-filtering strategy to evaluate and exclude low levels of microbial sequences, potential contaminations, and artifacts from plasma microbial 16S DNA sequencing analyses. Furthermore, we have applied our technique in three cohorts, including tobacco-smokers, HIV-infected individuals, and individuals with systemic lupus erythematosus (SLE), as well as corresponding controls. More than 97% of total sequence data was removed using stringent quality-filtering strategy analyses; those removed amplicon sequence variants (ASVs) were low levels of microbial sequences, contaminations, and artifacts. The specifically enriched pathobiont bacterial ASVs have been identified in plasmas from tobacco-smokers, HIV-infected individuals, and individuals with SLE but not from control subjects. The associations between these ASVs and disease pathogenesis were demonstrated. The pathologic activities of some identified bacteria were further verified *in vitro*. We present a quality-filtering strategy to identify pathogenesis-associated plasma microbiome. Our approach provides a method for studying the diagnosis of subclinical microbial infection as well as for understanding the roles of microbiome-host interaction in disease pathogenesis.

Keywords: plasma microbiome, quality-filtering strategies, contaminations and artifacts, pathogenic bacteria, microbiome-host interaction

INTRODUCTION

Blood and tissues were originally presumed to be sterile, and microbes were thought to occur only in cases of sepsis and live bacterial infections. However, recent research evidence has shown live bacteria in the blood and tissues which may play a role in the disease pathogenesis. Poore's group found cancer-specific microbial sequences in the tissues and the blood from different types of cancer in humans (Poore et al., 2020). In addition, another study led by Nejman's group found that distinct microbiome composition in seven cancer types and that the bacteria localized within both tumors and immune cells (Nejman et al., 2020). Microbe or microbial components were found in the blood from individuals with chronic inflammatory diseases (Potgieter et al., 2015; Lelouvier et al., 2016; Luo et al., 2019). Using bacterial 16S rRNA sequencing, Massier's group showed bacteria in the blood and adipose tissue samples, which were associated with increased tissue inflammation in obesity and type 2 diabetes (Massier et al., 2020). Moreover, the translocated microbial products were shown to induce immune perturbations and may contribute to some disease immunopathogenesis such as autoimmune diseases and central nervous system diseases (Niebauer et al., 1999; Brenchley et al., 2006; Klatt et al., 2010; Yoshimoto et al., 2013; Costa et al., 2016; Vieira et al., 2018). Our recent study showed that translocation of *Staphylococcus* promotes germinal center B cell activation and autoantibody production in mice and HIV+ individuals (Luo et al., 2019). All of these studies indicated that plasma or tissue microbiome might contribute to immune perturbations and disease pathogenesis.

However, investigation of plasma or tissue microbiome is highly challenging because of extremely low levels of bacterial biomass in the blood or tissues under a physiological condition, which leads to a high risk of contamination during procedures from plasma microbial cell-free DNA (cfDNA) isolation, amplification, to sequencing. Using whole-genome and whole-transcriptome sequencing, up to 92.3% of plasma microbiome sequence data was discarded after stringent decontamination to ensure valid results (Poore et al., 2020). Therefore, removing the background and artifacts from sequencing data is critical to obtain accurate results from the plasma microbiome analysis.

In this study, we applied a quality-filtering strategy to plasma microbiome to efficiently exclude amplicon sequence variants (ASVs) of contaminations and artifacts. After strict controls of contamination during plasma microbial DNA isolation, we found more than 97% of plasma microbial 16S DNA sequencing data resulted from low abundance and low prevalence of microbial sequences, contaminations, and artifacts, which was removed using stringent quality-filtering strategy analyses. Furthermore, we analyzed plasma microbiome in three cohorts, including tobacco-smoking individuals, individuals with HIV infection, or systemic lupus erythematosus (SLE), as well as corresponding control individuals.

MATERIALS AND METHODS

Subjects

Volunteers with SLE, HIV-infected individuals, and healthy individuals were recruited from the Medical University of South Carolina (MUSC) Lupus Clinic, Clinic of Infectious Diseases, and MUSC campus. The clinical characteristics are shown in **Supplementary Table S1**. All individuals with SLE met at least four American College of Rheumatology criteria for the classification of lupus as determined by the rheumatologist (Hochberg, 1997). The SLE clinical characteristics, disease manifestations, and medications being used are shown in **Supplementary Table S2**. HIV-infected individuals have been treated with antiretroviral therapy (ART) and have plasma HIV RNA below the limit of detection for at least 24 weeks (a single blip to ≤500 copies/ml was allowed). Tobacco-smoking and non-smoking control individuals were recruited from the Fifth Medical Center of Chinese PLA General Hospital in China. All tobacco-smokers were smoking for at least 5 years. This study was approved by each of the participating institutional review boards. All participants provided written informed consent. The tobacco-smoking cohort included 20 tobacco smokers and 21 non-smokers; the HIV cohort included 40 aviremic ART-treated HIV-infected individuals and 51 healthy controls; the SLE cohort included 19 women with SLE and 30 healthy control women. Venous blood was drawn into EDTA tubes and unstimulated saliva was collected after rinsing the mouth following a standard protocol (Topkas et al., 2012).

Plasma Microbial 16S rDNA Isolation

Fresh blood samples or endotoxin-free water (negative control, Catalog number: W50-640, LONZA, Walkersville, MD, United States) in EDTA-containing tubes (BD, San Jose, CA, United States) were centrifuged at 800 *g* for 15 min, which was followed by transferring the samples to new centrifuge tubes (Catalog number: 352098, BD). Plasma and water controls were placed in aliquots and stored at −80°C. DNA low-binding centrifuge tubes (Catalog number: 022431021, Eppendorf, Hamburg, Germany) were used to store the plasma samples. We avoided repeated freezing and thawing before microbial 16S rDNA isolation. Circulating bacterial DNA was extracted from 400 µl of plasma or the water control using the QIAamp UCP Pathogen Mini Kit (Catalog number: 50214, Qiagen, United States) according to the manufacturer's instructions.

16S rDNA Sequencing

Bacteria 16S rDNA was isolated from plasma and amplified using primers 515/806 with the barcode on the forward primer using a 35-cycle PCR. The HotStarTaq Plus Master Mix Kit (Catalog number: 203645, Qiagen) was used, and the 16S V4 variable region was amplified. To prevent batch-to-batch variation, all samples in each cohort were run concurrently. The PCR was performed under the following conditions: 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, and a final elongation step at 72°C for 5 min. The PCR products were checked on a 2% agarose gel to assess the amplification. Multiple samples were pooled together in equal concentrations and purified using Agencourt AMPure XP beads (Catalog number:

A63880, Beckman Coulter, Brea, CA, United States). The pooled and purified PCR products were used to prepare the DNA library according to the Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, United States) on a MiSeq platform following the manufacturer's guidelines.

Microbial 16S rDNA Data Processing

QIIME2[1] was applied to demultiplex the data generated by Illumina MiSeq sequencing into paired forward and reverse FASTQ. For each sample, barcodes and primers were depleted at paired sequences. Demultiplexed sequences were processed using the DADA2 (version 1.8; Callahan et al., 2016a) analysis pipeline in the R (https://www.r-project.org/, version 3.5.0) environment. Briefly, paired reads likely contain low quality throughout and pathological errors were truncated, and more than two expected errors per read were removed by paired reads (Edgar and Flyvbjerg, 2015). Unsupervised learning in DADA2 was performed to distinguish sequencing errors from real biological variation, while replacement and insertion of errors from the data were removed at the inference step. After merging the paired sequences, the chimera sequences were removed. ASVs were defined as clustering at 1% divergence (99% similarity), and the naive Bayesian classifier method was applied to assign taxonomy using the RDP v16 training set (Callahan et al., 2016b).

Plasma 16S Microbiome Analysis

ASV tables and different levels of taxonomic tables were imported into a phyloseq R package (version 3.7; McMurdie and Holmes, 2013) for statistical analysis. A 0.005% minimum abundance threshold was applied to exclude ASVs with a low abundance. Next, a prevalence filter was applied to ASVs, and the ASVs were retained only if the prevalence of specific ASV was more than 1/3 in any study group samples. A user-defined control filtering factor was applied to remove the potential contaminants and artifacts from the plasma microbiome. If the abundance of taxa from blank controls multiplied by the filtering factor was more than the abundance of taxa from plasma samples, the taxa were removed from samples. α-diversity of richness and evenness was calculated by the Simpson index of diversity in each sample. The Unifrac coefficient was calculated to evaluate β-diversity and compositional dissimilarity among the microbial community. The Multivariate Welch t-test was used to test the statistical significance of variances in microbiome composition between groups (Alekseyenko, 2016). Differential abundance testing between groups was compared by nonparametric Mann-Whitney's U tests at the OTU level. Values of p were adjusted for multiple comparisons by the Benjamin-Hochberg false discovery rate (FDR). The comparison analysis was performed using R.

[1]https://qiime2.org/

Autoantigen Microarray

Plasma levels of IgG autoantibodies were analyzed using 125-plex autoantigen arrays at the Genomics and Microarray Core, University of Texas Southwestern Medical Center. Plasma samples were treated with DNase I, diluted 1:50, and incubated with the autoantigen arrays. Cy3-labeled anti-human IgG was used to detect IgG autoantibodies. Mean fluorescence intensities (MFI) represent the signal intensity of each autoantibody.

Pro-inflammatory Cytokine Induction Stimulated by Disease-Enriched Bacteria *in vitro*

Peripheral blood mononuclear cells (PBMCs) from healthy individuals were isolated over a Ficoll-Hypaque cushion (GE, Pittsburgh, PA). After the bacteria were heat-inactivated at 60°C for 30 min, *Massilia timonae*, *Haemophilus parainfluenzae*, and *Anaerococcus prevotii* were added to cell culture at a final concentration of 5×10^6/ml; LPS from *Escherichia coli* 055:B5 was used as a positive control with a final concentration of 2 μg/ml. PBMCs were cultured with brefeldin A (5 μg/ml, BD) and incubated at 37°C for 6 h. Cells were then collected and washed with PBS, followed by a 20-min incubation with 50 μl aqua blue (Life Technologies, Carlsbad, CA, United States) at 4°C to exclude dead cells. Next, 50 μl of an antibody cocktail containing anti-human CD3 (OKT3), anti-human CD14 (M5E2), and anti-human CD16 (3G8) were used for surface staining. After washing and permeabilization, cells were intracellularly stained with anti-human TNF-α (MAb11), anti-human IL-1β (AS10), and anti-human IL-6 (MQ2-6A3). Fluorescence-labeled antibodies were purchased from BD or Biolegend (San Diego, CA). After washing, cells were collected and analyzed using a BD FACSVerse flow cytometer (BD). Data were analyzed using the FlowJo software (version 10.0.8).

RESULTS

16S rDNA Plasma Microbiome Analysis

Unlike saliva or feces, plasma has extremely low levels of bacterial DNA under a physiological condition but not a live infection. The external introduction of microbes or microbial DNAs through reagents and experimental procedures may cause artifacts in the plasma microbiome analyses. To study the source of potential artifacts, we investigated possible contaminations through the procedures of drawing blood, cfDNA isolation, and sequencing. Several controls were established to evaluate contaminations (**Supplementary Figure S1A**), as follows: (1) control i was the water control sequenced directly without cfDNA isolation; (2) control ii was the water control sequenced in isolated cfDNA; (3) control iii was the water control obtained through mimicking the whole procedure of drawing blood and plasma collection, cfDNA isolation, and sequencing. For this control, the skin of the antecubital fossa was wiped with 75% ethanol, and the sterile blood collection needle was used to puncture the skin but not into blood vessel. Next, the needle was withdrawn from the skin and, instead of drawing blood, the water in the eppendrof

tubes will be drawn into blood collection tubes; and (4) control iv was the water control used to assess possible contamination from the skin microbiota, for which the skin in the cubital fossa was wiped with 75% ethanol over areas of 3–4 cm^2 and allowed to dry. We added water to the "clean" skin, collected the water, and isolated and sequenced microbial cfDNA.

We first isolated microbial cfDNA from plasma of 25 healthy individuals. After 16 s rDNA sequencing, we performed the following steps: filtering and trimming of low-quality sequences, dereplication, merging of paired reads, and removal of chimeras. To determine whether the overall microbiome composition differed according to 16S isolation and sequencing, as well as within sample diversity (β-diversity), we conducted principal coordinate analysis (PCoA) based on unweighted UniFrac phylogenetic distances. There was no difference in the β-diversity of water control i, ii, and iii. However, the 16S rDNA isolated from control iv, obtained from the skin surface, showed differences in diversity when compared with the water controls i, ii, and iii ($p < 0.001$, Multivariate Welch *t*-test; **Supplementary Figure S1B**). Notably, the β-diversity of plasma microbial 16S rDNA was significantly different from those of the water controls ($p < 0.001$; **Supplementary Figure S1B**). This finding indicates that most artifacts occurred from the PCR and sequencing process. Although microbial 16S sequences in the water controls had backgrounds or contaminants, they were different from the 16S sequences of the plasma microbiome.

Removing Potential Artifacts and Contaminants From Plasma Microbiome

To exclude possible contaminant sequences and artifacts in the analysis, we performed a user-defined quality-filtering strategy (**Figure 1A**). We chose control iii as the blank control because it reflected the entire specimen handling process. A challenge for plasma microbiome analysis is the presence of heavy tail distribution and low prevalence of ASVs across samples. Therefore, before the removal of artifacts observed in plasma microbiome, a 0.005% minimum abundance threshold was applied to exclude ASVs with low abundance (Bokulich et al., 2013). In this case, ASVs with an abundance of less than 5 in each sample were removed; thus, 547 ASVs were excluded and 568 ASVs were retained after filtration.

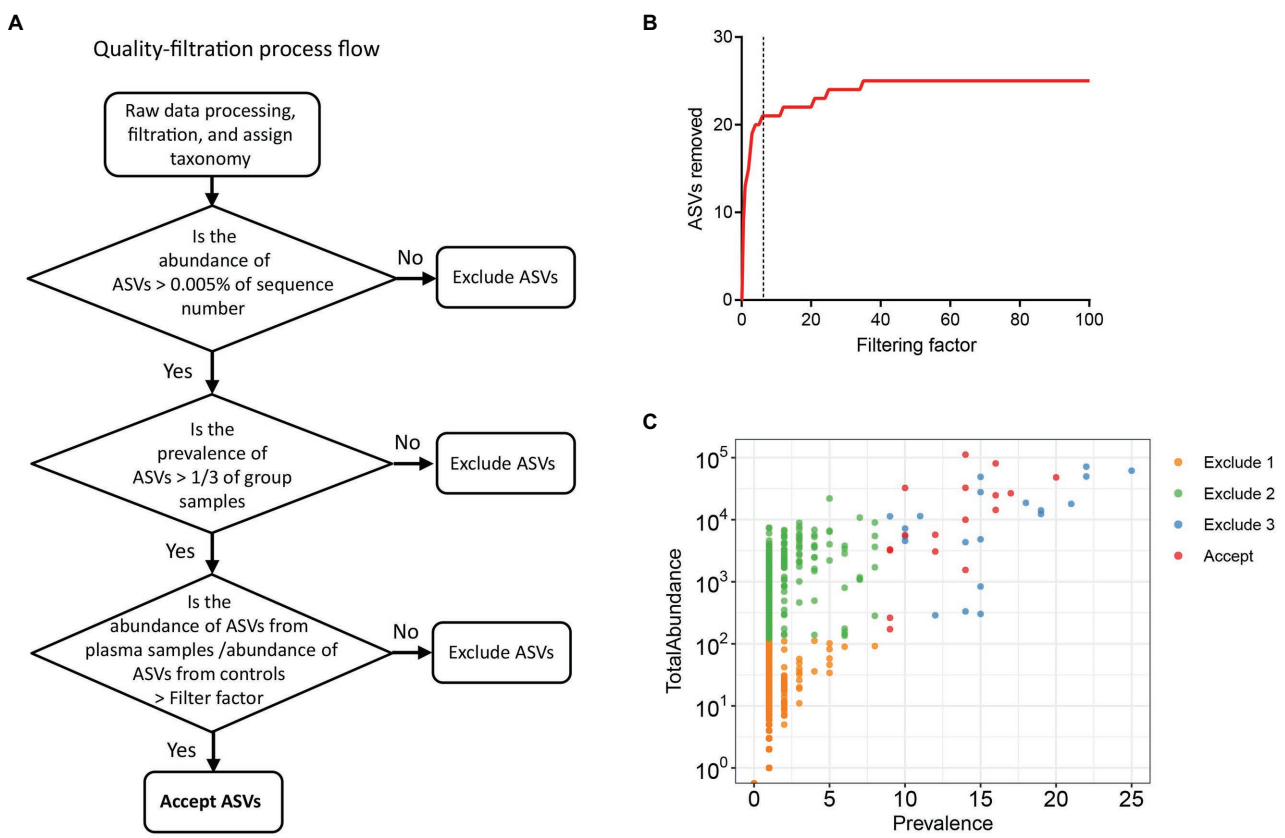

FIGURE 1 | Strategies for removing background and potential artifacts from plasma microbiome. **(A)** A workflow diagram of user-defined quality-filtering strategy to exclude the background and artifacts. **(B)** The number of removed amplicon sequence variants (ASVs) with different filtering factors; we chose the filtering factor of 6 which showed the lowest number of the plateau effect. **(C)** The abundance and prevalence of ASVs in each step of filtration. The "exclude 1" step is removing low abundance of ASVs across samples, in which a 0.005% minimum abundance threshold was applied. The "exclude 2" step is removing low prevalence of ASVs in each sample; we retained ASVs only if its prevalence was more than 1/3 across study groups. The "exclude 3" step is removing potential contaminants and artifacts using filtering factors.

The prevalence of ASVs in plasma microbiome of each donor may vary dramatically due to factors such as donor differences in the local microbiota communities and translocation from mucosal sites (e.g., oral cavity, gut, or vagina). Thus, to study the function of plasma microbiome in disease etiology or pathogenesis, it is essential to remove the interference from ASVs, which may exhibit a high abundance in some samples but a low prevalence across samples. To study the associations between enriched blood microbiome and disease pathogenesis, we excluded the ASVs shown in less than 1/3 individuals in either control or studied disease group (prevalence less than 1/3). After removing the low prevalence of ASVs, 527 ASVs were excluded and 41 remained.

Excluding all ASVs that appear in the controls may result in the loss of meaningful data, a filtering factor was applied to help ascertain whether the ASVs found in the control were due to cross-contamination from a real sample or from contaminations or artifacts by reagents during microbial DNA isolation and sequencing. If the taxa abundance in the water controls multiplied by the filtering factor was more than the taxa abundance in the plasma samples, the taxa present in the water control were considered contaminations from reagents or overamplification; thus, they were removed from the sample analyses. Otherwise, the taxa in the water control were considered cross-contamination from the plasma sample, and the taxa were retained. **Figure 1B** shows the number of removed ASVs when different filtering factors were applied. To exclude potential contaminants without loss of authentic information, we chose the filtering factor of six which showed the lowest number of the plateau effect (**Figure 1B**). A total of 16 ASVs were considered artifacts and removed from the samples, and 25 ASVs were accepted after filtration. The filtered-out ASVs from plasma microbiome varied from low to high abundance across the samples (**Figure 1C**).

Comparisons Between Plasma and Saliva Microbiome in Tobacco Smokers

Various disease states or altered microbiota composition may lead to a compromised mucosal epithelial barrier and translocation of microbial products into circulation. The use of tobacco is associated with the changes of oral microbiota and leads to a higher rate of periodontal diseases (Thomson et al., 2008). Translocation of oral bacteria into the blood has been implicated in the development of myocarditis, endocarditis, and cerebral infarction (Koren et al., 2011; Seringec et al., 2015). To investigate whether plasma microbiome is changed along with the mucosal microbiota, we have analyzed and compared the plasma and saliva microbiome from tobacco smokers and non-smoker controls. Blood and saliva samples were collected simultaneously for microbial 16S sequencing in 21 non-smokers and 20 tobacco smokers. In the saliva microbiome, we obtained 695 ASVs from 16S sequencing; after removing the ASVs with low abundance, 119 ASVs were retained from a total of 41 participants. In the plasma samples, as described above, after removing the ASVs with extremely low abundance and low prevalence, we removed potential contaminants using a filtering factor of 11 which was the lowest number of the plateau effect (**Figure 2A**); 42 ASVs were retained from 1,989 ASVs (2.1%) in 41 participants (**Figures 2B,C**).

There was no difference in the species diversity within each sample in the saliva from smokers and non-smokers based on the Gini Simpson (α-diversity) index (**Figure 2D**). However, the alpha-diversity of plasma microbiome in smokers was significantly higher compared with that of non-smokers (**Figure 2D**), suggesting that smoking may alter the oral microenvironment directly or indirectly through host immune responses to microbiota. In addition, unweighted UniFrac phylogenetic distances showed that tobacco smoking significantly altered the overall circulating microbiome composition (**Figure 2E**). In plasma samples, before removing the contaminants and ASVs with low prevalence, Axis 1 and Axis 2 of the PCoA interpreted 13.6 and 11% of the difference within samples, respectively; after filtering, Axis 1 and Axis 2 of the PCoA interpreted 35 and 13.4% of the difference within samples, respectively (**Figure 2E**).

To analyze the components of ASVs in the smoking and non-smoking groups, Benjamini and Hochberg FDR corrections were employed to adjust for multiple comparisons after performing nonparametric Mann-Whitney's *U* tests. In saliva samples, 25 ASVs were significantly different in smokers compared to non-smokers before adjusting for multiple comparisons, including 18 ASVs increased and 7 ASVs decreased in smokers compared to non-smokers (**Supplementary Table S3**). In plasma samples, 20 ASVs were different in smokers compared to healthy controls after adjusting with the FDR, and all taxa identified were enriched in the smokers (**Supplementary Table S4**). Among the taxa that were increased in the saliva of tobacco smokers, eight belonged to the *Streptococcus* family (**Supplementary Table S3**), which was also significantly enriched in the plasma microbiome of tobacco smokers (**Figures 2F,G**). *Streptococcus* was shown enrichment in both plasma and saliva from smokers and non-smokers, implying that some oral bacteria or bacterial products may translocate to the circulation. The shared microbiome in plasma and saliva could be a result of increased absolute numbers of specific microbiome or a preferential migration ability of certain taxa from altered oral or periodontal environment to the circulation in smokers.

Plasma Microbiome in HIV Disease

Previous studies from colleagues and our team have shown that HIV-infected individuals experience increased systemic microbial translocation even after long-term viral-suppressive ART (Brenchley et al., 2006; Jiang et al., 2009). To study the role of plasma microbiome in HIV disease pathogenesis, we performed microbial 16S sequencing in 40 aviremic ART-treated HIV+ individuals and 51 healthy controls. After removing ASVs with a low abundance and low prevalence from plasma microbiome, we used a filtering factor of 6 to remove potential contaminants and artifacts (**Figure 3A**); 24 ASVs were retained from 2,997 ASVs (0.80%) in ART-treated HIV+ subjects and healthy controls (**Figure 3B**). The overall circulating plasma microbial composition was

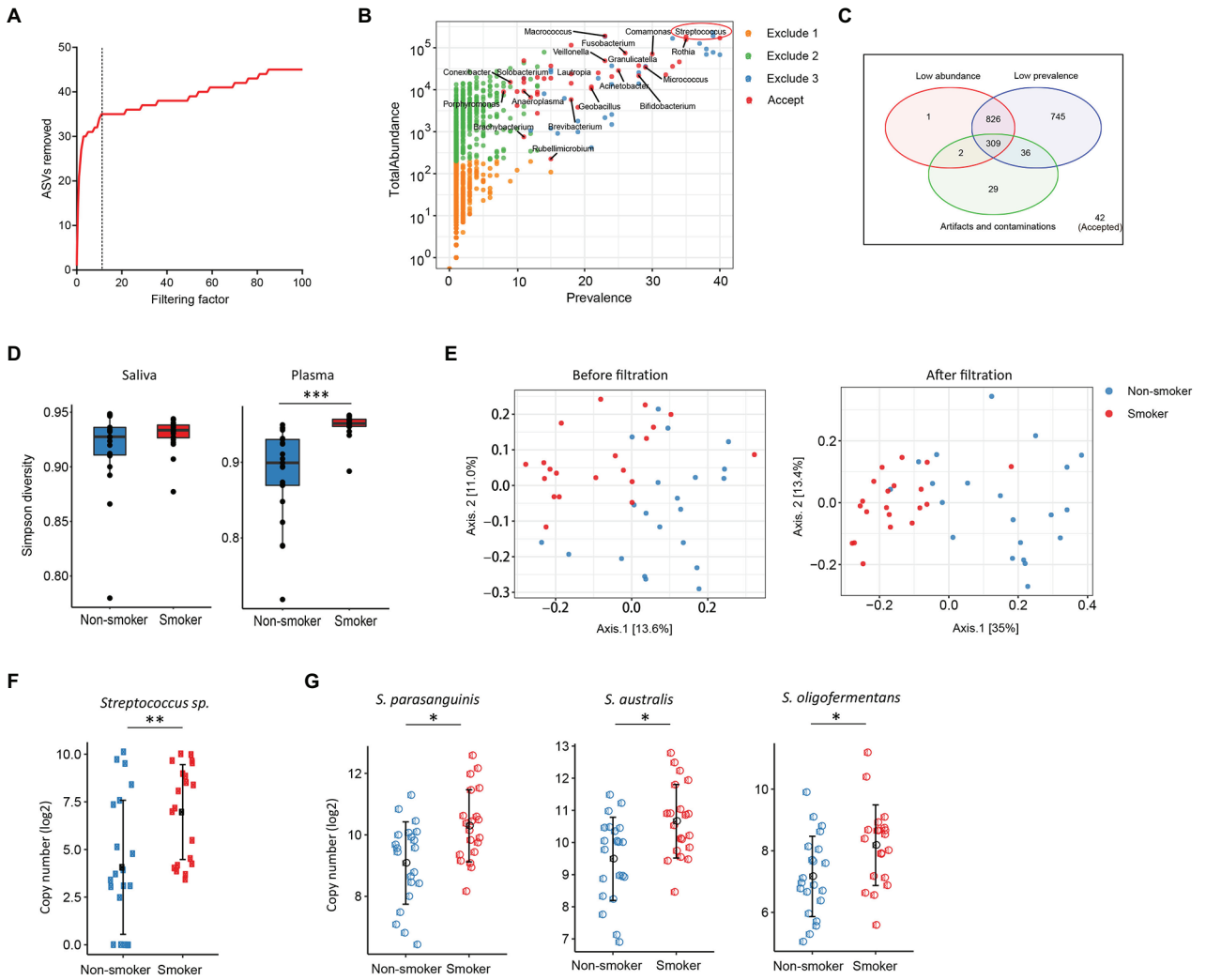

FIGURE 2 | Plasma and oral microbiome analyses in tobacco smokers compared with non-smokers. **(A)** The number of removed ASVs from plasma microbiome with different filtering factors; we chose the filtering factor of 11 which showed the lowest number of the plateau effect. **(B)** The abundance and prevalence of ASVs in each step of plasma microbiome filtration. The significantly different taxa in smokers compared with those in non-smokers are labeled in black (enriched in smokers). **(C)** Venn diagram showing the number and overlap of low abundance, low prevalence, and contaminations/artifacts in plasma microbiome. **(D)** The Gini Simpson diversity index (α-diversity) was used to compare the diversity of oral and plasma microbiome between smokers and non-smokers after filtration. **(E)** Principal coordinate analysis (PCoA) was conducted based on the unweighted UniFrac distance to determine the beta diversity of plasma microbiome before and after filtration. **(F)** *Streptococcus* was enriched in plasma microbiome of smokers compared to non-smokers. **(G)** Representative *Streptococcus* species in the oral microbiome of smokers and non-smokers. Mann-Whitney *U* (unpaired) and Spearman's rank tests. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

different between HIV-infected individuals and healthy controls (**Supplementary Figure S2A**; $p < 0.01$). Before filtering, Axis 1 and Axis 2 of the PCoA plot explained 9.2 and 7.2% of the differences within samples, respectively, compared to 37.7 and 15.2%, respectively, after filtering (**Supplementary Figure S2A**). Next, to analyze the components of the accepted 24 ASVs after filtering, 16 ASVs were significantly different between the two groups using FDR-adjusted values of *p* (**Supplementary Table S5**). After removing taxa that were not classified at the genus level, HIV-associated enrichment of plasma microbiome included *Veillonella*, *Massilia*, *Haemophilus*, *Arthrobacter*, and *Fusobacterium* genera (**Supplementary Figure S2B**), the top 3 enriched taxa in HIV+ individuals are shown in **Figure 3C**. In contrast, HIV-decreased plasma microbiome included *Altererythrobacter, Cryobacterium*, and *Anaerococcus* genera (**Supplementary Figures S2B,C**).

HIV-associated monocyte activation and chronic inflammation, defined by high levels of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, play a critical role in disease pathogenesis (Deeks, 2011). To evaluate whether the taxa enriched in plasmas of HIV+ individuals contribute to persistent inflammation in HIV disease, we tested the ability of heat-inactivated bacteria *M. timonae* and *H. parainfluenzae*

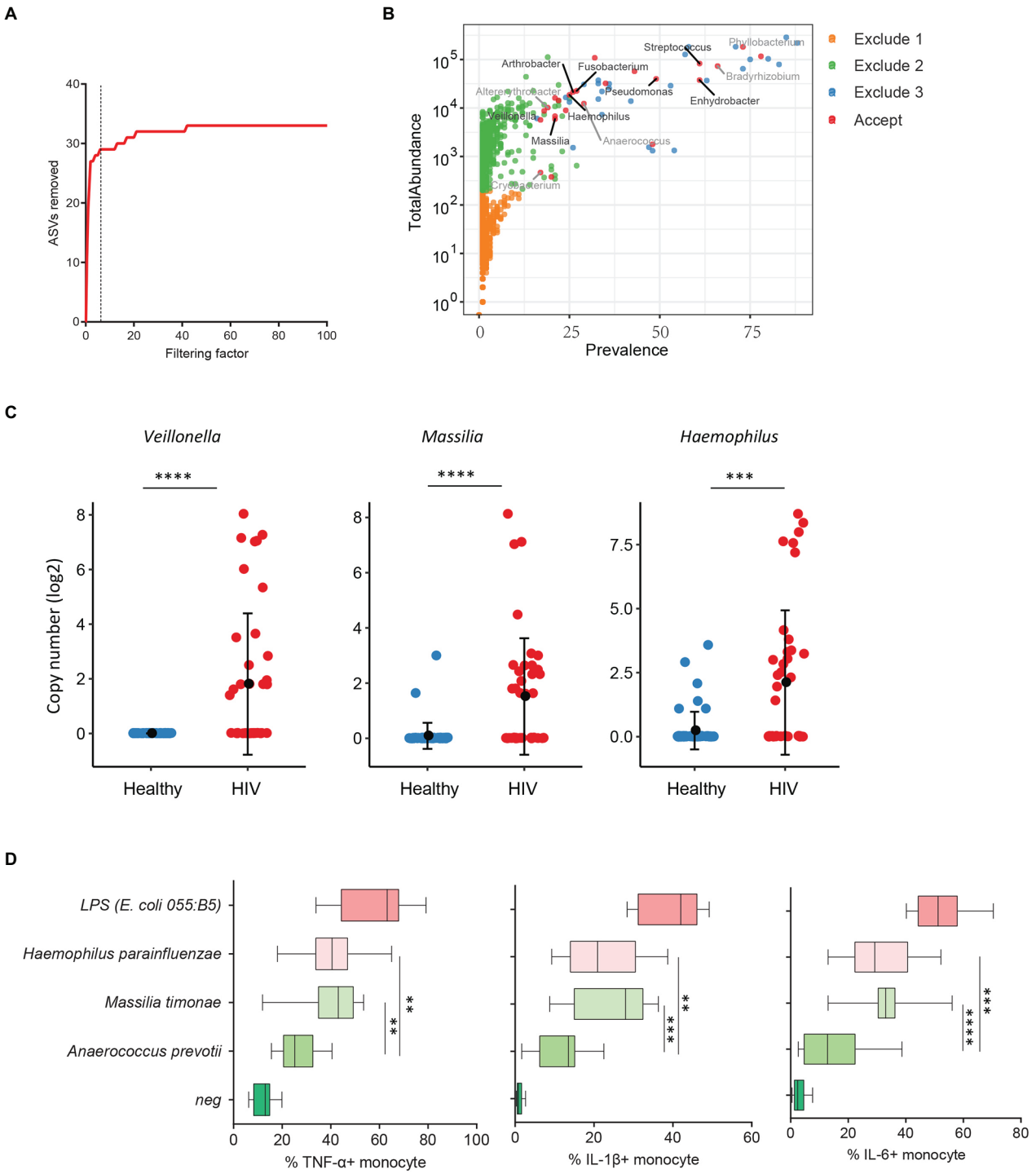

FIGURE 3 | Plasma microbiome in aviremic antiretroviral therapy (ART)-treated HIV+ individuals and healthy controls. **(A)** The number of removed ASVs with different filtering factors; we chose the filtering factor of 6 which showed the lowest number of the plateau effect. **(B)** The abundance and prevalence of ASVs in each step of plasma microbiome filtration are shown, and the significantly different taxa in HIV+ individuals compared with healthy controls are labeled in black (enriched in HIV+ individuals) or gray (decreased in HIV+ individuals). **(C)** The detected copies of the top three most increased taxa were shown in HIV+ individuals compared with healthy controls. **(D)** The percentages of TNF-α, IL-1β, and IL-6-producing monocytes in total monocytes were shown after peripheral blood mononuclear cells (PBMCs) were stimulated with heat-inactivated bacteria (5×10^6 units/ml) or 2 μg/ml of LPS ($n = 15$, healthy individuals). Cytokines were measured in monocytes using flow cytometry after 6 h of stimulation. Box and whisker plots (Min. to Max.) show the responses of monocytes to each bacterium or LPS. Nonparametric Mann-Whitney's *U* tests and one-way ANOVA test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{***}p < 0.0001$.

to induce pro-inflammatory cytokines. Notably, we observed a decrease in *Anaerococcus* sp. in the plasma microbiome of HIV+ individuals compared to controls. *Anaerococcus prevotii* is the commercial for available commensal bacterium within *Anaerococcus* genus which is a commonly isolated flora found on the skin and in the oral cavity (Murphy and Frick, 2013). Therefore, *A. prevotii* was chosen as a negative control bacterium. LPS from *E. coli* 055:B5 was chosen as a positive control. Human PBMCs from 15 healthy individuals were stimulated with *M. timonae*, *H. parainfluenzae*, *A. prevotti*, or LPS for 6 h, and TNF-α, IL-1β, and IL-6 production in monocytes were evaluated using flow cytometry. Treatment of both *M. timonae* and *H. parainfluenzae* but not *A. prevotii* increased the percentages of TNF-α, IL-1β, and IL-6-producing monocytes compared to monocytes in medium controls ($p < 0.05$; **Figure 3D**). This result suggests that *M. timonae* and *H. parainfluenzae* enriched in the plasmas of HIV+ individuals may play a role in the persistent inflammation in aviremic ART-treated HIV disease.

Plasma Microbiome in Systemic Lupus Erythematosus

Increasing evidence has revealed a link between increased levels of microbial translocation and autoimmune disease pathogenesis (Fasano, 2012; Mu et al., 2017). To study the association of plasma microbiome and autoimmune disease pathogenesis, 30 healthy women and 19 women with SLE were assessed for the plasma microbiome analysis. All 49 plasma samples were from premenopausal women. After removing low abundance and low prevalence ASVs, a filtering factor of 3 was used to remove potential contaminants (**Figure 4A**); 21 ASVs were retained from 2,189 ASVs (0.95%) in the healthy controls and SLE patients (**Figure 4B**). Ten out of the 21 ASVs showed significant differences in SLE patients compared with healthy controls after FDR adjustment, which were enriched in SLE patients (**Supplementary Table S6**). After removing taxa that were not classified at the genus level, the retained taxa enriched in SLE patients are presented (**Figure 4C**).

Systemic lupus erythematosus is characterized by loss of tolerance to self-antigens and autoantibody production (Tsokos et al., 2016). To determine the link between plasma microbiome and SLE disease pathogenesis, we examined the association of plasma microbiome with plasma autoantibody reactivities to a panel of 125 autoantigens in SLE patients and healthy controls. Notably, enriched genera of *Desulfoconvexum, Desulfofrigus, Desulfovibrio, Draconibacterium, Planococcus*, and *Psychrilyobacter* in SLE patients were directly correlated with increased plasma levels of various autoantibodies (**Figure 4D** and **Supplementary Table S7**), including representative SLE-related IgG autoantibodies, such as anti-double-stranded DNA (anti-dsDNA), anti-nucleosome, anti-histone, and anti-collagen antibodies (**Figure 4D**; Schett et al., 2002; Reveille, 2004). Most of these microbiome-associated autoantibodies target nuclear and cell-matrix antigens, which are common host self-antigens previously identified in SLE and other autoimmune diseases. These results suggest that alterations of plasma microbiome are associated with autoantibody production in SLE disease. We also tested the ability of heat-inactivated bacteria *Planococcus* to induce pro-inflammatory cytokines. Treatment of *Planococcus citreus* increased the percentages of TNF-α, IL-1β, and IL-6-producing monocytes compared to monocytes in medium controls ($p < 0.05$; **Figure 4E**). This result suggests that *Planococcus*, related to autoantibody production in SLE, may play a role in the inflammation in SLE disease.

DISCUSSION

The microbial translocation, including microbiota or microbial fragments, may directly interact with immune cells to remodel immune responses. The blood microbiome has been reported to associate with pathogenesis in a variety of infectious and noninfectious diseases (Clement et al., 2005; Nielsen et al., 2012; Sato et al., 2014; Puri et al., 2018; Poore et al., 2020). However, due to the extremely low levels of microbial biomass in the plasma under physiologic conditions and artifacts during microbial DNA isolation and the sequencing, analysis of blood microbiome is challenging. Although cautions should be taken during the isolation of plasma microbial DNAs to avoid potential contaminations, artifacts from microbial 16S sequencing are still observed and should be controlled in the analysis. In this study, we provided a guideline for filtering out potential contaminations and artifacts in a plasma microbiome analysis and applied to study pathogenesis of various diseases as examples.

Before removing the artifacts from plasma microbiome, we filtered out the ASVs with a low abundance and low prevalence. Extremely low levels of ASVs likely have a minor or nonexistent effect on the immune system. Thus, a 0.005% minimum abundance threshold has been applied in the current study, suggested from a previous study (Bokulich et al., 2013). A larger amount of ASVs with a low abundance across the samples may be a result of microbial or microbial product translocation across a permeable mucosa (e.g., gut). ASVs with a low abundance and low prevalence were removed, and only ASVs with a prevalence of more than 1/3 in any study group were retained. However, the distinct species and taxa may play similar functions in the individual microbiome. Therefore, taxa unique to individuals may play similar roles in disease pathogenesis. Removing any sequence that is not in at least 1/3 of the cohort may also filter out some meaningful taxa related to the disease. To exclude the potential artifacts, controls are critical because they reflect contaminations during the process of blood collection until 16S sequencing, the taxa detected in the water controls should be removed from the experimental samples at the ASV levels. However, the removal of ASVs should be performed with cautions, because some ASVs present in the controls may originate from cross-contamination with the experimental samples during DNA isolation, PCR, or sequencing. Due to the lack of plasma 16S templates available to compete for amplification reagents in the PCR and sequencing stages, the background or contaminant ASVs in each control likely will have a similar or higher copy number than those in the plasma samples. In this case, a filtering factor was applied to remove potential contaminants

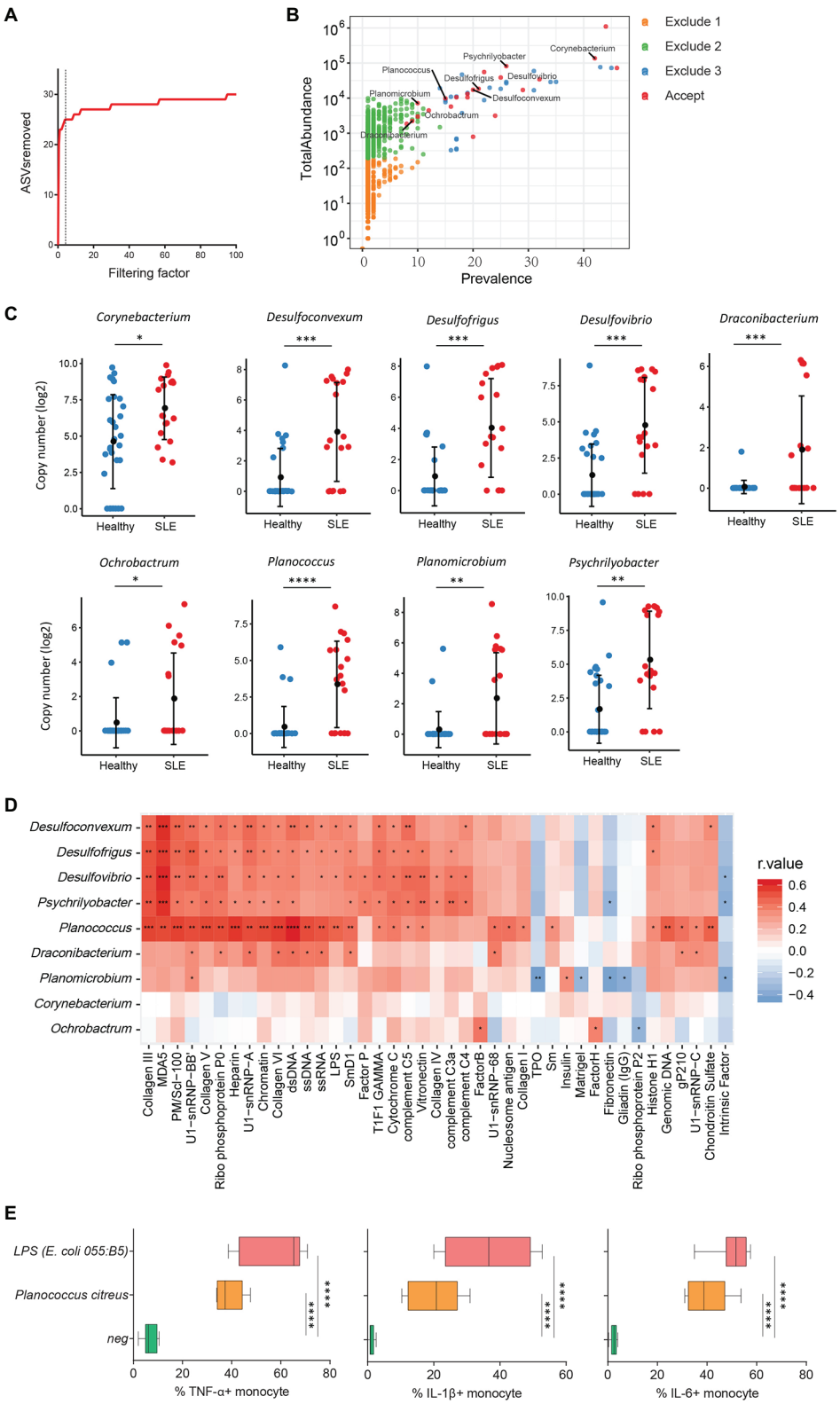

FIGURE 4 | Plasma microbiome in female systemic lupus erythematosus (SLE) patients and healthy female controls. **(A)** The number of removed ASVs with different filtering factors; we chose the filtering factor of 3 which showed the lowest number of the plateau effect. **(B)** The abundance and prevalence of ASVs in

(Continued)

FIGURE 4 | each step of plasma microbiome filtration are shown, and the significantly different taxa in SLE patients compared with healthy controls are labeled in black (enriched in SLE patients). **(C)** The significantly different taxa in SLE patients compared with healthy controls. **(D)** Correlations between plasma levels of autoantibodies and differential plasma taxa levels in the two study groups. Correlation coefficient *r* values are indicated by color from red (directly correlations) to blue (inversely correlations); *p*-value significance is shown in the heatmap. **(E)** The percentages of TNF-α, IL-1β, and IL-6-producing monocytes in total monocytes were shown after PBMCs were stimulated with heat-inactivated bacteria (5×10^6 units/ml) or 2 μg/ml of LPS ($n = 7$, healthy individuals). Cytokines were measured in monocytes using flow cytometry after 6 h of stimulation. Box and whisker plots (Min to Max) show the responses of monocytes to each bacterium or LPS. Nonparametric Mann-Whitney's *U* and Spearman's rank tests and one-way ANOVA test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

from plasma microbiome, whereby we only remove ASVs with abundance in experimental samples with less than a factor of times of the corresponding abundance in the water controls. The choice of this filtering factor is a processing choice for which we suggest as a rule of thumb to examine the filtering plateau effect and to make a determination regarding which ASVs may be retained. Using this filtering strategy, we observed that 97–99% of the ASVs in the plasma microbiome data were accounting for extremely low abundance, low prevalence, contamination, or artifacts and should be excluded in analyses.

Next, we performed plasma microbiome analyses in tobacco-smoking individuals as an example. Smoking has been associated with an altered oral microbiota and periodontal diseases (Mason et al., 2015; Yu et al., 2017), which may facilitate the translocation of some oral microbiota into the blood. Consistent with a previous study (Wu et al., 2016), we discovered an enrichment of *Streptococcus* in the saliva microbiota from smokers compared to non-smokers (**Figure 2G**). Importantly, *Streptococcus* was also found enriched in the plasma microbiome from smokers compared to non-smokers (**Figure 2F**). Although many other taxa were enriched in the saliva of smokers, they were not enriched in the paired plasma samples, suggesting that *Streptococcus* may have a stronger migration ability from smoking-associated oral mucosa to the circulation. *Streptococcus* in the blood has been found to play a role in atherosclerosis and cardiovascular diseases (Dos Reis et al., 1982; Brown et al., 2014; Jackson et al., 2016).

We also analyzed plasma microbiome in HIV and SLE as well as the link between plasma microbiome and disease pathogenesis. After the implementation of our filtration guidelines to remove the potential contaminants and ASVs with a low abundance, we noted significant differences in the β-diversity and composition of plasma microbiome between HIV+ subjects and healthy controls. Most of the taxa enriched in HIV+ individuals in this study are well-known pathogenic bacteria. *Arthrobacter* spp. and *M. timonae* have been isolated from blood, cerebrospinal fluid, and bone of clinical patients with infectious diseases or end-organ diseases (Lindquist et al., 2003; Mages et al., 2008). Moreover, *H. parainfluenzae* is an opportunistic pathogen (Smith et al., 1976); we found that *in vitro* treatment with HIV-enriched heat-inactivated *M. timonae* and *H. parainfluenzae* induced robust inflammatory responses in human monocytes (**Figure 3D**). SLE is a systemic autoimmune disease, and autoantibodies play a key role in disease pathogenesis. However, the underlying etiology and mechanisms of autoantibody production in SLE are not fully understood (Graham and Utz, 2005). Persistent immune activation induced by the translocation of microbial components from the gastrointestinal tract or other mucosal sites into the circulation has been considered one of the predisposing factors in SLE (Brenchley et al., 2006; Brenchley and Douek, 2012; Ogunrinde et al., 2019). In this study, we found that some SLE-enriched taxa had a direct correlation with plasma autoantibody levels (**Figure 4D**). The functional evaluation of these SLE-enriched taxa in lupus disease merits further investigation.

CONCLUSION

In summary, we provide a strategy for filtering potential contaminants and backgrounds to analyze plasma microbiome, which provides a promising method to study translocated bacteria or bacteria in tissues in various diseases and their roles in modulating systemic immune responses and disease pathogenesis.

DATA AVAILABILITY STATEMENT

The full plasma microbiome sequencing data sets can be available at https://www.ncbi.nlm.nih.gov/sra/PRJNA551477.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Volunteers with SLE, HIV-infected individuals and healthy individuals were recruited from the Medical University of South Carolina (MUSC) Lupus Clinic, Clinic of Infectious Diseases, and MUSC campus. This study was approved by each participating institutional review boards. All participants provided written informed consent. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZLu and WJ designed the project. ZLu analyzed plasma microbiome next-generation sequencing data and wrote the manuscript. ZLu, AA, and ML contributed to data interpretation and discussion. ZLu, EO, Q-ZL, ML, LH, DK, and JO performed experiments. WJ, AA, EO, Q-ZL, BT, ZLi, and GG contributed to the study design and revised 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/fmicb.2020.613268/full#supplementary-material

REFERENCES

Alekseyenko, A. V. (2016). Multivariate Welch t-test on distances. *Bioinformatics* 32, 3552–3558. doi: 10.1093/bioinformatics/btw524

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

Brenchley, J. M., and Douek, D. C. (2012). Microbial translocation across the GI tract. *Annu. Rev. Immunol.* 30, 149–173. doi: 10.1146/annurev-immunol-020711-075001

Brenchley, J. M., Price, D. A., Schacker, T. W., Asher, T. E., Silvestri, G., Rao, S., et al. (2006). Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat. Med.* 12, 1365–1371. doi: 10.1038/nm1511

Brown, A. O., Mann, B., Gao, G., Hankins, J. S., Humann, J., Giardina, J., et al. (2014). *Streptococcus pneumoniae* translocates into the myocardium and forms unique microlesions that disrupt cardiac function. *PLoS Pathog.* 10:e1004383. doi: 10.1371/journal.ppat.1004383

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016a). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Callahan, B. J., Sankaran, K., Fukuyama, J. A., McMurdie, P. J., and Holmes, S. P. (2016b). Bioconductor workflow for microbiome data analysis: from raw reads to community analyses. *F1000Res.* 5:1492. doi: 10.12688/f1000research.8986.2

Clement, S., Vaudaux, P., Francois, P., Schrenzel, J., Huggler, E., Kampf, S., et al. (2005). Evidence of an intracellular reservoir in the nasal mucosa of patients with recurrent *Staphylococcus aureus* rhinosinusitis. *J. Infect. Dis.* 192, 1023–1028. doi: 10.1086/432735

Costa, F. R., Françozo, M. C., De Oliveira, G. G., Ignacio, A., Castoldi, A., Zamboni, D. S., et al. (2016). Gut microbiota translocation to the pancreatic lymph nodes triggers NOD2 activation and contributes to T1D onset. *J. Exp. Med.* 213, 1223–1239. doi: 10.1084/jem.20150744

Deeks, S. G. (2011). HIV infection, inflammation, immunosenescence, and aging. *Annu. Rev. Med.* 62, 141–155. doi: 10.1146/annurev-med-042909-093756

Dos Reis, G., Gaspar, M., and Barcinski, M. (1982). Immune recognition in the streptococcal carditis of mice: the role of macrophages in the generation of heart-reactive lymphocytes. *J. Immunol.* 128, 1514–1521.

Edgar, R. C., and Flyvbjerg, H. (2015). Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics* 31, 3476–3482. doi: 10.1093/bioinformatics/btv401

Fasano, A. (2012). Leaky gut and autoimmune diseases. *Clin. Rev. Allergy Immunol.* 42, 71–78. doi: 10.1007/s12016-011-8291-x

Graham, K. L., and Utz, P. J. (2005). Sources of autoantigens in systemic lupus erythematosus. *Curr. Opin. Rheumatol.* 17, 513–517. doi: 10.1097/01.bor.0000171215.87993.6b

Hochberg, M. C. (1997). Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* 40:1725. doi: 10.1002/art.1780400928

Jackson, M. A., Goodrich, J. K., Maxan, M.-E., Freedberg, D. E., Abrams, J. A., Poole, A. C., et al. (2016). Proton pump inhibitors alter the composition of the gut microbiota. *Gut* 65, 749–756. doi: 10.1136/gutjnl-2015-310861

Jiang, W., Lederman, M. M., Hunt, P., Sieg, S. F., Haley, K., Rodriguez, B., et al. (2009). Plasma levels of bacterial DNA correlate with immune activation and the magnitude of immune restoration in persons with antiretroviral-treated HIV infection. *J. Infect. Dis.* 199, 1177–1185. doi: 10.1086/597476

Klatt, N. R., Harris, L. D., Vinton, C. L., Sung, H., Briant, J. A., Tabb, B., et al. (2010). Compromised gastrointestinal integrity in pigtail macaques is associated with increased microbial translocation, immune activation, and IL-17 production in the absence of SIV infection. *Mucosal Immunol.* 3, 387–398. doi: 10.1038/mi.2010.14

Koren, O., Spor, A., Felin, J., Fåk, F., Stombaugh, J., Tremaroli, V., et al. (2011). Human oral, gut, and plaque microbiota in patients with atherosclerosis. *Proc. Natl. Acad. Sci.* 108 (Suppl. 1), 4592–4598. doi: 10.1073/pnas.1011383107

Lelouvier, B., Servant, F., Paisse, S., Brunet, A. C., Benyahya, S., Serino, M., et al. (2016). Changes in blood microbiota profiles associated with liver fibrosis in obese patients: a pilot analysis. *Hepatology* 64, 2015–2027. doi: 10.1002/hep.28829

Lindquist, D., Murrill, D., Burran, W. P., Winans, G., Janda, J. M., and Probert, W. (2003). Characteristics of Massilia timonae and Massilia timonae-like isolates from human patients, with an emended description of the species. *J. Clin. Microbiol.* 41, 192–196. doi: 10.1128/JCM.41.1.192-196.2003

Luo, Z., Li, M., Wu, Y., Meng, Z., Martin, L., Zhang, L., et al. (2019). Systemic translocation of Staphylococcus drives autoantibody production in HIV disease. *Microbiome* 7:25. doi: 10.1186/s40168-019-0646-1

Mages, I. S., Frodl, R., Bernard, K. A., and Funke, G. (2008). Identities of Arthrobacter spp. and Arthrobacter-like bacteria encountered in human clinical specimens. *J. Clin. Microbiol.* 46, 2980–2986. doi: 10.1128/JCM.00658-08

Mason, M. R., Preshaw, P. M., Nagaraja, H. N., Dabdoub, S. M., Rahman, A., and Kumar, P. S. (2015). The subgingival microbiome of clinically healthy current and never smokers. *ISME J.* 9, 268–272. doi: 10.1038/ismej.2014.114

Massier, L., Chakaroun, R., Tabei, S., Crane, A., Didt, K. D., Fallmann, J., et al. (2020). Adipose tissue derived bacteria are associated with inflammation in obesity and type 2 diabetes. *Gut* 69, 1796–1806. doi: 10.1136/gutjnl-2019-320118

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Mu, Q., Kirby, J., Reilly, C. M., and Luo, X. M. (2017). Leaky gut as a danger signal for autoimmune diseases. *Front. Immunol.* 8:598. doi: 10.3389/fimmu.2017.00598

Murphy, E. C., and Frick, I. -M. (2013). Gram-positive anaerobic cocci–commensals and opportunistic pathogens. *FEMS Microbiol. Rev.* 37, 520–553. doi: 10.1111/1574-6976.12005

Nejman, D., Livyatan, I., Fuks, G., Gavert, N., Zwang, Y., Geller, L. T., et al. (2020). The human tumor microbiome is composed of tumor type–specific intracellular bacteria. *Science* 368, 973–980. doi: 10.1126/science.aay9189

Niebauer, J., Volk, H. -D., Kemp, M., Dominguez, M., Schumann, R. R., Rauchhaus, M., et al. (1999). Endotoxin and immune activation in chronic heart failure: a prospective cohort study. *Lancet* 353, 1838–1842. doi: 10.1016/S0140-6736(98)09286-1

Nielsen, H. H., Qiu, J., Friis, S., Wermuth, L., and Ritz, B. (2012). Treatment for helicobacter pylori infection and risk of Parkinson's disease in Denmark. *Eur. J. Neurol.* 19, 864–869. doi: 10.1111/j.1468-1331.2011.03643.x

Ogunrinde, E., Zhou, Z., Luo, Z., Alekseyenko, A., Li, Q. Z., Macedo, D., et al. (2019). A link between plasma microbial translocation, microbiome, and autoantibody development in first-degree relatives of systemic lupus erythematosus patients. *Arthritis Rheum.* 71, 1858–1868. doi: 10.1002/art.40935

Poore, G. D., Kopylova, E., Zhu, Q., Carpenter, C., Fraraccio, S., Wandro, S., et al. (2020). Microbiome analyses of blood and tissues suggest cancer diagnostic approach. *Nature* 579, 567–574. doi: 10.1038/s41586-020-2095-1

Potgieter, M., Bester, J., Kell, D. B., and Pretorius, E. (2015). The dormant blood microbiome in chronic, inflammatory diseases. *FEMS Microbiol. Rev.* 39, 567–591. doi: 10.1093/femsre/fuv013

Puri, P., Liangpunsakul, S., Christensen, J. E., Shah, V. H., Kamath, P. S., Gores, G. J., et al. (2018). The circulating microbiome signature and inferred functional metagenomics in alcoholic hepatitis. *Hepatology* 67, 1284–1302. doi: 10.1002/hep.29623

Reveille, J. D. (2004). Predictive value of autoantibodies for activity of systemic lupus erythematosus. *Lupus* 13, 290–297. doi: 10.1191/0961203303lu1015oa

Sato, J., Kanazawa, A., Ikeda, F., Yoshihara, T., Goto, H., Abe, H., et al. (2014). Gut dysbiosis and detection of "live gut bacteria" in blood of Japanese patients with type 2 diabetes. *Diabetes Care* 37, 2343–2350. doi: 10.2337/dc13-2817

Schett, G., Smole, J., Zimmermann, C., Hiesberger, H., Hoefler, E., Fournel, S., et al. (2002). The autoimmune response to chromatin antigens in systemic lupus erythematosus: autoantibodies against histone H1 are a highly specific marker for SLE associated with increased disease activity. *Lupus* 11, 704–715. doi: 10.1191/0961203302lu247oa

Seringec, N., Guncu, G., Arihan, O., Avcu, N., and Dikmenoglu, N. (2015). Investigation of hemorheological parameters in periodontal diseases. *Clin. Hemorheol. Microcirc.* 61, 47–58. doi: 10.3233/CH-141892

Smith, C., Kanner, R., Golden, C., and Renzetti, A. (1976). Haemophilus influenzae and Haemophilus parainfluenzae in chronic obstructive pulmonary disease. *Lancet* 307, 1253–1255. doi: 10.1016/S0140-6736(76)91733-5

Thomson, W. M., Poulton, R., Broadbent, J. M., Moffitt, T. E., Caspi, A., Beck, J. D., et al. (2008). Cannabis smoking and periodontal disease among young adults. *JAMA* 299, 525–531. doi: 10.1001/jama.299.5.525

Topkas, E., Keith, P., Dimeski, G., Cooper-White, J., and Punyadeera, C. (2012). Evaluation of saliva collection devices for the analysis of proteins. *Clin. Chim. Acta* 413, 1066–1070. doi: 10.1016/j.cca.2012.02.020

Tsokos, G. C., Lo, M. S., Costa Reis, P., and Sullivan, K. E. (2016). New insights into the immunopathogenesis of systemic lupus erythematosus. *Nat. Rev. Rheumatol.* 12, 716–730. doi: 10.1038/nrrheum.2016.186

Vieira, S. M., Hiltensperger, M., Kumar, V., Zegarra-Ruiz, D., Dehner, C., Khan, N., et al. (2018). Translocation of a gut pathobiont drives autoimmunity in mice and humans. *Science* 359, 1156–1161. doi: 10.1126/science.aar7201

Wu, J., Peters, B. A., Dominianni, C., Zhang, Y., Pei, Z., Yang, L., et al. (2016). Cigarette smoking and the oral microbiome in a large study of American adults. *ISME J.* 10, 2435–2446. doi: 10.1038/ismej.2016.37

Yoshimoto, S., Loo, T. M., Atarashi, K., Kanda, H., Sato, S., Oyadomari, S., et al. (2013). Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* 499, 97–101. doi: 10.1038/nature12347

Yu, G., Phillips, S., Gail, M. H., Goedert, J. J., Humphrys, M. S., Ravel, J., et al. (2017). The effect of cigarette smoking on the oral and nasal microbiota. *Microbiome* 5:3. doi: 10.1186/s40168-016-0226-6

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REVIEW
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Intestinal Bacteria Encapsulated by Biomaterials Enhance Immunotherapy

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The human intestine contains thousands of bacterial species essential for optimal health. Aside from their pathogenic effects, these bacteria have been associated with the efficacy of various treatments of diseases. Due to their impact on many human diseases, intestinal bacteria are receiving increasing research attention, and recent studies on intestinal bacteria and their effects on treatments has yielded valuable results. Particularly, intestinal bacteria can affect responses to numerous forms of immunotherapy, especially cancer therapy. With the development of precision medicine, understanding the factors that influence intestinal bacteria and how they can be regulated to enhance immunotherapy effects will improve the application prospects of intestinal bacteria therapy. Further, biomaterials employed for the convenient and efficient delivery of intestinal bacteria to the body have also become a research hotspot. In this review, we discuss the recent findings on the regulatory role of intestinal bacteria in immunotherapy, focusing on immune cells they regulate. We also summarize biomaterials used for their delivery.

Keywords: intestinal bacteria, probiotic, immunotherapy, immune cell, biomaterial, oral delivery

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INTRODUCTION

In the human intestine, there are more than 100 trillion bacterial cells that mainly exhibit commensalism with the host (1, 2) and play a role in the maintenance of host health (3). In this commensalistic relationship, intestinal bacteria not only participate in the regulation of the host immune system and the promotion of bone marrow hematopoiesis but also regulate the maturation and function of hematopoietic cells originating from the yolk sac (4, 5). Moreover, intestinal bacteria can regulate the barrier function *via* interaction with epithelial cells and stromal cells (6–8). The functions of intestinal bacteria widely range from local to systemic levels, including metabolism regulation, hematopoiesis, inflammation, immunity, and other physiological functions (8–11). However, changes in intestinal ecology can disrupt this commensalistic relationship. For instance, some symbiotic bacteria called pathobionts might cause, or even worsen, a number of diseases (10, 12, 13). Increasing evidence has demonstrated that dysbiosis has been connected to various diseases, including tumors, viral infection, inflammatory bowel disease (IBD), diabetes, and liver cirrhosis (10, 14, 15).

Owing to their impact on various human diseases, intestinal bacteria have been recently receiving increasing attention. Aside from their pathogenic effects, intestinal bacteria also exert

beneficial effects in reducing gastrointestinal inflammation, preventing colorectal cancer, and treating some diseases (16–19). The mechanisms by which intestinal bacteria affect inflammation, immunity, and local therapeutic response have also been elucidated (8, 20, 21). Currently, intestinal bacteria, including *Escherichia coli* (22, 23), *Bifidobacterium* (24, 25), *Filamentous fungus* (10), *Lactobacillus* (26), *Bacillus subtilis* (27), and *Bacteroides fragilis* (28, 29), have been applied in the therapy of diseases such as diabetes (30), gastrointestinal diseases (31, 32), and allergic diseases (33, 34). Interestingly, intestinal bacteria can also potentiate antitumor therapies (35–37). With the advancements in precision medicine, intestinal bacteria have thus become increasingly important in the treatment of various diseases. Thus, studies aiming to understand the factors influencing intestinal bacteria and the strategies for their manipulation to enhance therapeutic efficacy are increasing (36).

Traditional tumor therapies, such as chemotherapy, surgery, radiation therapy, and molecular targeted therapy, are the primary methods used to treat tumors at different stages (31). However, they have some drawbacks, such as toxic side effects and recurrence after treatment. With the advancement in tumor research, immunotherapy has emerged as a promising therapeutic modality (38). Compared with chemotherapy, immunotherapy of tumors has fewer side effects. In cases wherein immunotherapy is effective in patients suffering from tumors, it could prolong their survival period and even cure tumors clinically. Although immunotherapy has shown promising potential in the treatment of hematological and solid tumors, its efficacy remains limited due to the variability in immune responses and susceptibilities to tumor types among patients (39). Therefore, only some patients benefit from immunotherapy. Recent studies have shown that the regulation of intestinal bacteria can influence the effects of immunotherapy (35, 40, 41). Moreover, intestinal bacteria have been applied to the immunotherapy of many diseases, such as type 1 diabetes and IBD, and yielded remarkable outcomes (42, 43). This treatment approach that uses intestinal bacteria is called bacterial therapy.

To delineate the factors that affect intestinal bacteria and improve their immunotherapeutic effects, many studies have attempted to regulate intestinal bacteria using antibiotic treatment or fecal microbiota transplantation (FMT) (14, 44, 45). However, both methods can cause large-scale and holistic changes in abundance and diversity of intestinal bacteria. Therefore, to regulate the population of a certain bacterium or transfer some type of probiotics into the host for bacterial therapy, an efficient way of delivering intestinal bacteria is necessary. Compared with intravenous (i.v.) injection, the oral administration of bacteria can improve patient compliance and avoid the risk of systemic infections that might be caused by the i.v. injection. However, because the oral administration route involves the passage of bacteria through the stomach and gut, various bacterial activities may be compromised due to the presence of gastric acid and bile salts in the gastrointestinal system. Therefore, the design and selection of biomaterials for the encapsulation and delivery of intestinal bacteria are essential. Many biomaterials, including Eudragit (46), chitosan (47), and alginate (48), have been widely used to encapsulate and protect intestinal bacteria against acid, bile, and other harsh components. Moreover, the properties of these biomaterials, including permeability, mechanical stability, and pH sensitivity, have been specially designed to improve the survival of intestinal bacteria in the acidic environment of the stomach and ensure their complete release in the intestine (49, 50).

In this review, we focused on the immunotherapeutic effects of intestinal bacteria mainly exerted by regulating various immune cells, as well as the biomaterials used to encapsulate and deliver the bacteria possessing these regulatory functions. We first discuss various immune cells, which are classified according to their types, regulated by intestinal bacteria. Then, we summarize the recent progress on biomaterial encapsulation methods in various intestinal bacterial species. **Figure 1** schematically shows the oral administration of intestinal bacteria and their immunoregulatory function in various diseases.

REGULATING IMMUNOTHERAPY BY AFFECTING IMMUNE CELLS

The immune system of the human body has a crucial impact on the development of various diseases. Diseases, such as inflammation and tumors, can alter the microenvironment of the body. For example, tumor cells in the tumor microenvironment (TME) have been reported to closely interact with the extracellular matrix (ECM) and stromal cells (51). A variety of immune and nonimmune cells that secrete cytokines and chemokines and express various surface receptors have been found in the TME. These cells have been shown to drive chronic inflammation and immunosuppression and promote the development of proangiogenic tumor environment (52). Immune cells, such as lymphocytes, dendritic cells (DCs), macrophages, and natural killer (NK) cells, are important in tumor development or suppression (53). A spatiotemporal dynamic analysis of 28 different kinds of immune cells that infiltrated tumors found that the composition of infiltrating immune cells changes at each tumor stage, with particular cells having a major impact on survival (54). Therefore, regulating these immune cells may improve the immunotherapy of tumors and other diseases.

Owing to some limitations in the application of immunotherapy (39, 55) and the regulation of the intestinal bacterial function (56, 57), many studies have combined the use of intestinal bacteria with immunotherapy, revealing their key role in regulating the response of immunotherapy, especially in tumors, by affecting immune cells (58–61). The findings of recent studies on bacteria used for immunotherapy and the immune cells they influenced are summarized in **Table 1**.

Regulation of T-Cells by Intestinal Bacteria

T-lymphocytes, also called T-cells, are derived from the bone marrow. Following their differentiation and development in the thymus, they are distributed to immune organs and tissues through the blood circulatory and lymphatic systems, thus

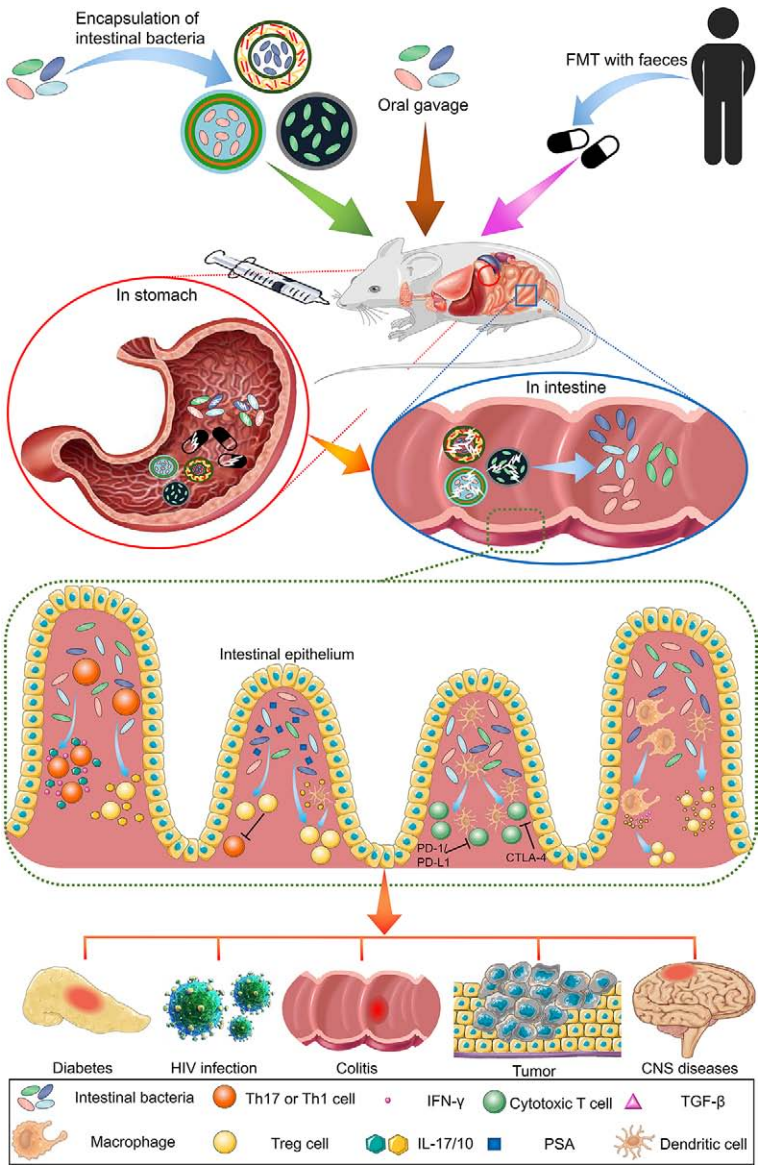

FIGURE 1 | Overview of the oral administration of intestinal bacteria for immunotherapy in various diseases. Common intestinal bacterial delivery methods include oral delivery (gavage) and intravenous injection. Gavage is more widely used because of its safety profile. Compared with free bacteria and FMT, bacteria encapsulated by biomaterials can resist the acidic environment of the stomach, and their contents can be released in the intestine. The released bacteria exert immune regulation functions beneficial to the treatment of various diseases.

exerting their immune function. T-cells are critical in many diseases, including tumors (75). According to their function and surface markers, T-cells can be classified as helper T-cells, regulatory T-cells, cytotoxic T-cells, suppressor T-cells, and memory T-cells. In this section, we summarized the recent findings on the regulation of these T-cells by intestinal bacteria.

Helper T-Cells

Helper T-cells, whose major surface marker is CD4, play a role in intermediate immune response. They can proliferate and spread to activate other types of immune cells involved in direct immune response. Helper T-cells can be activated through an antigenic reaction with a polypeptide presented by the major histocompatibility complex II (MHC II). Once activated, they can secrete cytokines, regulate, or assist in the immune response.

Intestinal bacteria regulate immunotherapy for type 1 diabetes (T1D) *via* helper T-cells. A study analyzing the fecal samples of biobreeding diabetes-prone (BB-DP) and biobreeding diabetes-resistant (BB-DR) mice (56) found that the fecal matter of BB-DR mice is enriched in both *Lactobacillus* and *Bifidobacterium* species, whereas that of BB-DP mice is abundant in *Bacteroides*. The prevalence of *Bacteroides* in mice with T1D suggests that

TABLE 1 | Intestinal bacteria used for the immunotherapy of diseases.

Bacterial species	Disease	Immune cell target	Reference
Lactobacillus	Type 1 diabetes	Th17 cells	(56)
Bifidobacteria			
segmented filamentous bacteria (SFB)	Diabetes	Th17 cells	(62)
Lactobacillus johnsonii strain N6.2 (LjN6.2)	Type 1 diabetes	Th17 cells	(63)
Lactobacillus casei	HIV infection	Th1 cells	(64)
Lactobacillus rhamnosus GG (LGG)	Liver cancer	Th17 cells	(16)
Escherichia coli Nissle 1917 (EcN)			
heat-inactivated VSL#3			
Bacteroides	Inflammation	Tregs	(65)
Bacteroides fragilis	IBD	$Foxp3^+$ Tregs	(43)
L.paracasei DSM 13434,	Inflammation	$Foxp3^+$ Tregs	(66)
L.plantarum DSM15312 and DSM 15313			
L.acidophilus, L.casei,	Inflammation	$Foxp3^+$ Tregs	(67)
Lactobacillus reuteri, Bifidobacteria			
Streptococcus thermophilus			
Faecalibacterium spp.	Melanoma	Cytotoxic T cells	(68)
Bifidobacteria longum	Metastatic melanoma	Cytotoxic T cells	(24)
Collinsella aerofaciens			
Enterococcus faecium			
Enterococcus hirae 13144 *(E. hirae)*	Tumor	Memory T cells	(45)
Bacteroides fragilis	Tumor	Memory T cells	(44)
Bifidobacterium	Tumor	Dendritic cells	(15)
Bifidobacteria LMG 13195	Inflammation	Dendritic cells	(69)
Bacteroides fragilis	IBD	Dendritic cells	(70)
Bacillus subtilis 7025	Tumor	Macrophages	(27)
SCFA	CNS diseases	Microglia	(71)
DNA of *Escherichia coli*	Tumor	B cells	(72, 73)
DNA of *Mycobacteria*	Tumor	NK cells	(74)

intestinal bacteria are involved in the occurrence and development of diseases. Furthermore, intestinal bacteria can regulate T-helper 17 (Th17) cells. Among non-obese diabetic (NOD) mice, female mice without segmented filamentous bacteria (SFB) showed a higher prevalence of diabetes, whereas those with SFB were resistant to diabetes (62). However, in male mice, there was no significant difference in the onset of diabetes between the two groups. To explore the relationship between SFB and diabetes, flow cytometry was performed on tissue derived from the small intestinal lamina and associated lymph nodes in SFB^+ and SFB^- female mice. The results showed an evident induction of Th17 cells in the small intestinal lamina propria of SFB^+ females. Another study has demonstrated that the oral administration of the *Lactobacillus johnsonii* strain N6.2 (LjN6.2) from BB-DR rats conferred T1D resistance to BB-DP rats, but that of *Lactobacillus reuteri* strains did not (76). This resistance of LjN6.2-fed BB-DP mice was due to a change in Th17 cells within the mesenteric lymph nodes (63), which was not observed in non-gut–draining axillary lymph nodes, indicating that the change in Th17 cells was caused by LjN6.2 interactions within the mesenteric lymph node. Overall, these studies indicate that the induction of T1D could be circumvented by the intestinal bacterial-mediated differentiation of Th17 cells.

Most importantly, by affecting helper T cells, the intestinal bacteria can regulate immunotherapy for tumors. To investigate the potential mechanism underlying the inhibition of tumor progression and control of hepatocellular carcinoma (HCC) by intestinal bacteria feeding, Li and coworkers used Prohep to design their experiments (16). Prohep is a new mixture of intestinal bacteria comprising heat-inactivated VSL#3, viable *Escherichia coli* Nissle 1917 (EcN), and *Lactobacillus rhamnosus* GG (LGG) (1:1:1). Prohep not only significantly reduced tumor size and weight by 40% but also retarded tumor growth compared with the control (**Figures 2A, B**). In addition, after treatment with Prohep, the hypoxic region of the tumor was obviously increased, indicating that the reduction in tumor size might have been associated with cell death caused by hypoxia (**Figure 2C**). In **Figure 2D**, the confocal Z-stacks of 3D models show that compared with the control group, the number of vessel sprouts and the region of blood vessels in each tumor section were obviously decreased. The tumor group treated with intestinal bacteria had decreased number of Th17 cells. Moreover, metagenome sequencing revealed the association between intestinal bacteria and HCC development. Therefore, treatment with Prohep may promote the growth of beneficial bacterial colonies, including *Oscillibacter* and *Prevotella*, which are known producers of anti-inflammatory metabolites. Subsequently, these bacteria led to the reduction in the number of Th17 cells and promoted the differentiation of regulatory T-cells (Tregs; introduced in next section) in the intestine. In summary, tumor reduction induced by probiotic feeding relies on the downregulation of IL-17 and its major producer, Th17 cells.

Intestinal bacteria have also been found to prevent infections and suppress allergies. Chilba et al. demonstrated that a co-culture with Lactobacillus casei *in vitro* promoted the development of Th1 cells, resulting in an increased production of interferon γ (IFN-γ), and also stimulated CD11b+ cells to produce interleukin 12 (IL-12) in mouse spleen cells (77).

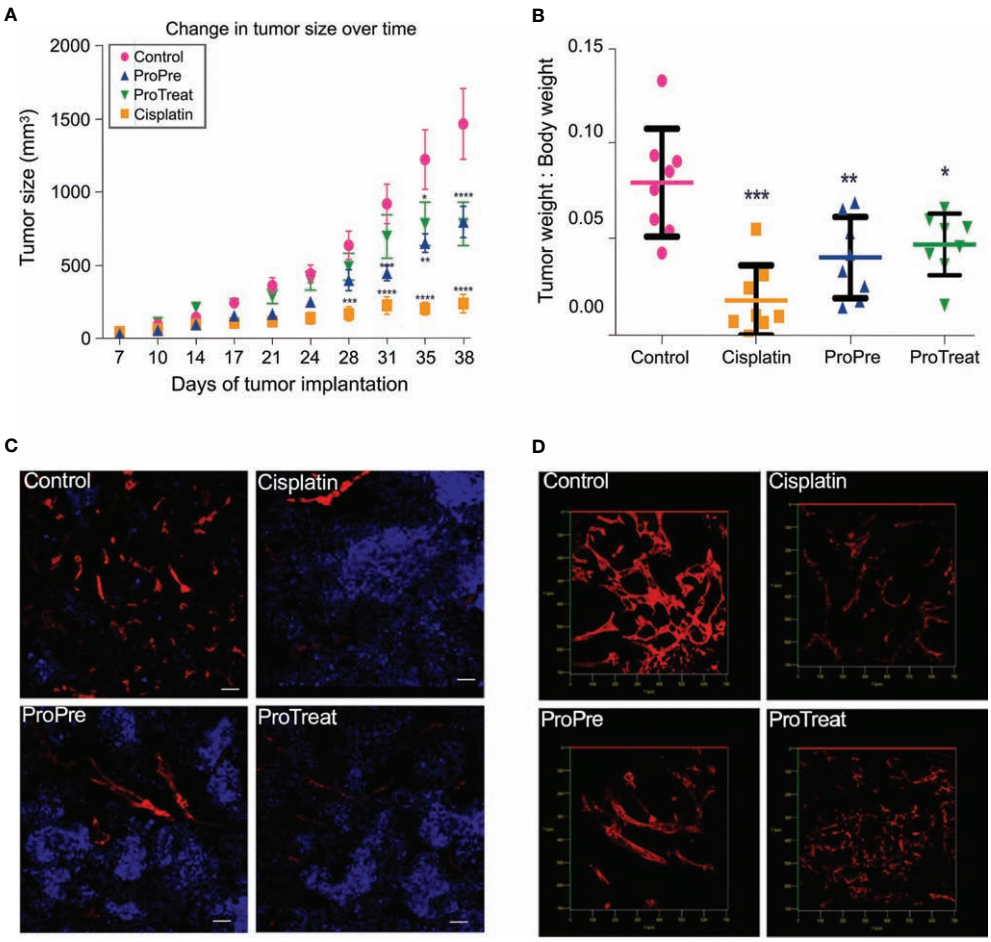

FIGURE 2 | The Prohep intestinal bacterial mixture improves hypoxia in tumor tissues and reduces tumor volume. **(A)** Changes in tumor size during 38 d. **(B)** Tumor weight of each group after the experiment. **(C)** Immunostaining of representative tumor sections using the CD31 angiogenesis (red) and GLUT-1 hypoxia (blue) markers. **(D)** Images of a 3D model acquired after superimposing multiple confocal planes by confocal Z-stack imaging (section thickness of 25 μm). *0.01 < *P* value < 0.05; **0.001 < *P* value < 0.01; ****P* value < 0.001. Adapted from ref. (16).

Intestinal bacteria that increase the production of IL-12, such as *L. casei*, can effectively prevent infections. By controlling the balance of Th1/Th2 cells, *L. casei* has also been reported to suppress allergic diseases. This immunomodulation is partly due to the regulation of indoleamine 2,3-dioxygenase (IDO). IDO is widely distributed in the body and participates in tryptophan metabolism to produce 3-hydroxyanthranilic, quinolinic acid, and kynurenine (78). These three tryptophan degradation products have excellent blood–brain barrier penetration ability, which is a key obstacle to the application of many drugs to treat brain diseases (79). Moreover, 3-hydroxyanthranilic acid and quinolinic acid can selectively drive the apoptosis of Th1 cells, whereas kynurenines can promote the development of Tregs, suggesting that these metabolites may possess immunoregulatory effects (80). By expressing IDO and activating subsequent biological processes, the use of specific intestinal bacteria could also enhance the efficacy of immunotherapy for brain diseases.

Tregs

Tregs exhibit immunosuppressive functions, and their deficiency contributes to the development of allergies and autoimmune diseases; thus, they are T-cells that regulate autoimmune activity *in vivo*. In addition, Tregs expressing specific tumor-associated antigens have been found in patients with tumor and aggregate in several kinds of solid tumors, where they may play a role in protecting tumors from cytotoxic immune responses (81). In fact, there is a negative correlation between the survival rate of patients with tumors and Treg infiltration. Therefore, the regulation of Tregs could be a promising treatment strategy in many diseases.

Bacterial metabolites affect the occurrence and progression of inflammation *via* mediating the communication between intestinal bacteria and the immune system. For example, butyrate, a short-chain fatty acid (SCFA) secreted by intestinal bacteria, promotes the extrathymic production of Tregs (21), indicating that intestinal bacteria exhibit a regulatory function in

the immunotherapy of inflammatory diseases, such as IBD. In a prospective study of patients suffering from metastatic melanoma treated with ipilimumab, Dubin et al. compared the composition of preinflammation fecal microbiota before and after the development of colitis (65). Increased *Bacteriodes* were found in the stools of participants resistant to colitis, whereas enriched *Faecalibacterium* and other *Firmicutes* were detected in those with high incidence of checkpoint-block-induced colitis (57). Moreover, enrichment in the genus *Bacteroides* was observed in patients with resistant colitis. As one of the primary bacterial species in the human gut, *Bacteroides* can suppress inflammation *via* stimulating the differentiation of Tregs (82). This immunomodulatory effect was consistent with the results obtained by Dubin et al. and Chaput et al. (57, 65).

In addition, immunotherapy can also be regulated by specialized bacterial molecules, such as the polysaccharide A (PSA) of *Bacteroides fragilis*. Round et al. demonstrated that the Toll-like receptor (TLR) pathway could be activated in T-cells by the human commensal bacterium *Bacteroides fragilis* to establish an interaction between the host and intestinal bacteria (28). Deficiency in TLR2, which is located on $CD4^+$ T-cells, can promote antimicrobial immune responses, resulting in reduced colonization of the mucosa by *B. fragilis*. However, this can be recovered by the PSA of *B. fragilis* that directly activates TLR2 on $Foxp3^+$ regulatory T-cells, leading to the production of mucosal tolerance. Meanwhile, *B. fragilis* not expressing PSA could not suppress the host immune response. Another study confirmed the regulatory function of the PSA of *B. fragilis* and showed that *B. fragilis* induced the development of $Foxp3^+$ Tregs with unique inducible hereditary characteristics (43). Unlike naturally produced Tregs, these inducible Tregs were found in peripheral tissues, such as the intestine, rather than in thymic tissues and can secrete cytokines, such as interleukin 10 (IL-10) (83). Round et al. found that germ-free animals colonized with *B. fragilis* had increased inhibitory capacity of Tregs and produced only anti-inflammatory cytokines from $Foxp3^+$ T-cells in the intestine (43). In addition, they showed that as an immunomodulatory molecule, the PSA of *B. fragilis* induced $CD4^+$ T-cells into inducible $Foxp3^+$ Tregs. These processes required TLR2 signaling to induce Tregs and express IL-10. More importantly, the PSA of *B. fragilis* was demonstrated to not only prevent but also cure experimental colitis in animals, supporting that intestinal bacterial molecules not only mediate the balance between health and disease but also regulate the efficacy of immunotherapy for many diseases.

After recognizing that changes in the composition of intestinal bacteria can influence inflammation, the potential mechanisms of intestinal dysfunction following HIV-1 infection became clearer. Early in 1990, researchers found that most patients with acquired immunodeficiency syndrome (AIDS) exhibited gastrointestinal disorders of variable severity (84); thus, they assumed that maintaining the integrity of intestinal ecology could improve the clinical outcomes of patients suffering from AIDS. Consequently, increasing evidence showed that probiotic treatment can protect the intestinal surface, delay the progression of HIV-1 infection to AIDS, and provide specific benefits in patients with HIV-1 infection (64, 85). The balance between Th17 cells and Tregs is an important immunoregulatory mechanism that influences the production of functional host immune responses to infection (86, 87). This balance is mediated by tryptophan catabolites, tryptamine, indole-3-aldehyde (IAld), indoleacetic acid, and indolelactic acid (ILA), which act as aryl hydrocarbon receptor (AHR) ligands and promote the differentiation of naive $CD4^+$ helper T-cells into Tregs (88). Intestinal bacteria, such as *Lactobacilli* (89) and *Clostridium sporogenes*, can metabolize and convert tryptophan into the above-mentioned AHR ligands, thereby affecting the Th17/Tregs balance and exerting a beneficial effect on autoimmune diseases and inflammation. More specifically, intestinal bacteria inducing the differentiation of Th17 cells or upregulating Tregs can suppress the progression of inflammation and provide benefits in patients with viral infection. In addition, by expressing IDO and producing tryptophan metabolites, intestinal bacteria can activate the AHR signaling pathway and induce IL-22 production (90). IL-22 mainly fulfills intestinal barrier function and maintains the intestinal homeostasis by mediating mucosal host defense, thereby helping the body resist the invasion of intestinal pathogenic bacteria (91, 92). Overall, intestinal bacteria can indirectly up-regulate IL-22 to counter pathogenic bacterial infections and diarrhea caused by gut dysbiosis.

Kwon et al. identified a mixture of probiotics, designated as IRT5, comprising *L. casei*, *L. acidophilus*, *Bifidobacterium bifidium*, *L. reuteri*, and *Streptococcus thermophilus* (67). After its oral administration for 20 days, IRT5 upregulated $CD4^+$ $Foxp3^+$ Tregs and induced the hypo-responsiveness of T-cells. In addition, the transformation of T-cells into Foxp3+ Tregs was shown to be directly mediated by DCs (discussed in Section of DCs) specialized to express IDO as well as suppressor cytokines such as TGF-β and IL-10.

By regulating Tregs, intestinal bacteria can also affect the immunotherapy of allergic diseases, such as allergic asthma. Russelle et al. showed that changes in the intestinal bacteria due to antibiotics increase the susceptibility of neonatal mice to allergic asthma (93). Compared with streptomycin, vancomycin reduced intestinal bacterial diversity, especially that of *Bacteroides* and *Clostridiales*, and the reduced *Bacteroides* were replaced by abundant *Lactobacilli*. As previously discussed, species under the genus *Bacteroides*, such as *B. fragilis*, are associated with T-cell differentiation. Additionally, treatment with vancomycin decreased the number of $Foxp3^+$ Tregs in the colon, but not in the lungs, following the destruction of *Clostridiales* population. Atarashi et al. reached the same conclusion and identified the *Clostridium* species as potent inducers of Tregs (94). However, the role of *Lactobacilli* remains controversial as the said study showed its negative correlation with Tregs, whereas another study showed that it induces Tregs (95). Therefore, there might be other unknown factors participating in the regulation of immunotherapy by intestinal bacteria or an unknown interaction between other *Lactobacilli* species or strains. The specific mechanisms, however, remain unexplored. As there are thousands of intestinal bacteria in the body, maintaining commensalism with the host, all intestinal bacteria in each organ should be seen as a whole when we regulate

intestinal bacteria in order to influence immunotherapy. Based on this view, FMT has been applied at the microbial level to alter immune responses in the body and has shown good results in the regulation of immunotherapy.

Cytotoxic T-Cells

Cytotoxic T-cells are specific T-cells that secrete various cytokines and participate in immune function. They exert a killing effect on certain viruses, tumor cells, and other antigenic substances. In particular, cytotoxic T-cells and NK cells play an important role in defense against viruses and tumors. Recently, cytotoxic T-cells regulated by intestinal bacteria has been associated with the efficacy of the immune checkpoint inhibitors (ICIs).

ICI therapy leads to the activating of T-lymphocyte-mediated immune responses by inhibiting the interaction between T-cell inhibitory receptors and their homologous ligands on stromal cells or tumors (96). Currently, ICIs are mainly monoclonal antibodies targeting the programmed cell death protein 1 (PD-1)/PD ligand 1 (PD-L1) axis and cytotoxic T-lymphocyte antigen 4 (CTLA-4), and ICIs have achieved great success in the immunotherapy of tumors. However, ICIs cannot suppress tumor progression in most patients; thus, they often result in immune-related adverse events (irAEs) (97). The use of antibiotics could reduce the efficacy of ICIs in tumor immunotherapy, with ICIs showing excellent efficacy in the presence of specific intestinal bacterial species (98). For example, the immunostimulatory and antitumor effects of the CTLA-4 blockade was demonstrated to depend on the presence of various *Bacteroides* gut species (99). In patients suffering from renal cell carcinoma (RCC), advanced-stage non–small cell lung cancer (NSCLC), or bladder tumor treated with PD-1/PD-L1 blockade, the use of broad-spectrum antibiotics shortly before or after treatment was associated with adverse clinical outcomes (45).

Increasing evidences have demonstrated that intestinal bacteria can influence the effect of immune checkpoint blockade (ICB) treatment by regulating cytotoxic T-cells. Gopalakrishnan et al. observed notable differences in the composition and diversity of intestinal bacteria between responsive and non-responsive (NR) patients with melanoma receiving anti-PD-1 immunotherapy as shown in **Figure 3A** (68). Responsive patients had increased abundance of the *Ruminococcaceae* family (including *Faecalibacterium* spp.) compared to NR, and enrichment in *Faecalibacterium* spp. positively correlated with progression-free survival (PFS) and cytotoxic T-cell infiltration in the TME. To study the mechanism, FMT results confirmed the transferability of this phenotype. The detailed experimental design is presented in **Figure 3B**. Slower tumor growth and better immunotherapy efficacy were observed in mice subjected to FMT with stool samples from the responsive patients than in those from NR (**Figures 3C, D**). In a separate study, Matson and coworkers verified these findings using pretreated fecal samples from 42 patients with metastatic melanoma (24). *Enterococcus faecium*, *Collinsella aerofaciens*, and *Bifidobacterium longum* were found to be more enriched in responders. In addition, transferring fecal samples from the responders to germ-free mice has been shown to improve tumor control, enhance T-cell responses, and increase anti-PD-1 therapeutic effect. These effects are due to the regulation of cytotoxic T-cells by these intestinal bacteria.

Memory T-Cells

Memory and effector T-cells are formed following the division and differentiation of T-cells, respectively. Memory T-cells are of vital importance in recurrent immune responses. When the host immune system is invaded by the same antigen, memory T-cells remobilize the mechanisms used to kill the antigen previously. The identity of memory T-cells can be determined *via* the expression of CD45RA, CD27, and CD62L (100). Currently, memory T-cells are used in vaccines for infectious diseases (101) and have recently been found to exert an anti-tumor function.

Routy et al. found a relationship among *Akkermansia muciniphila*, *Enterococcus hirae* 13144 (*E. hirae*), and memory T-cells (45). As shown in **Figure 4A**, patients with NSCLC and RCC responding to the PD-1/PD-L1 blockade had increased abundance of *A. muciniphila* in their feces than non-responders. They next investigated the responses of memory T-cells in the peripheral blood to the microbiota after initiating PD-1 blockade. **Figure 4B** presents the response of circulating memory $CD4^+$ and $CD8^+$ T-cells collected from 27 patients with NSCLC and 28 with RCC subjected to anti-PD-1 treatment. Due to the function of *A. muciniphila* and *E. hirae*, the responses of memory Th1 and Tc1 cells were enhanced in the responders, resulting in increased production of IFN-γ (**Figure 4C**). **Figure 4D** shows that based on 32 fecal samples, *E. hirae* was more abundant in responders with NSCLC than in non-responders. This further supports the relevance of probiotics, such as *E. hirae* and *A. muciniphila*, in predicting efficacious treatment.

In addition, Vetizou and coworkers demonstrate the modulatory function of intestinal bacteria in responses to anti-CTLA-4 therapy by regulating of memory T-cells (44). In both mice and humans, T-cell specific response to *B. fragilis* or *B. thetaiotaomicron* was related to the effect of the CTLA-4 blockade. Tumors in germ-free (GF) or antibiotic-treated mice did not respond to CTLA-4 blockade. However, after the adoptive transfer of *B. fragilis*-specific T-cells or gavage with *B. fragilis*, the presence of *Bacteroides* species determined the antitumor effect of the CTLA-4 blockade. Subsequently, the dynamics of the response of memory T-cells in humans and mice during CTLA-4 blockade was analyzed and revealed that T-cell response to anti-CTLA-4 therapy was due to the production of Th1 cells induced by specific memory T-cells. Moreover, the adoptive transfer of this specific type of memory T-cells into GF or patients with antibiotic-treated tumors could partially restore the efficacy of the immune checkpoint blocker.

Regulation of Antigen Presenting Cells by Intestinal Bacteria

Antigen presenting cells (APCs) can absorb, process, and present antigens; hence, they constitute a key part of innate immunity. The major histocompatibility complex (MHC) class II molecule expressed on the surface of APCs can absorb pathogen proteins and help process them into short peptide segments, which are presented to T-cells. Thus, APCs are also known as the initiators

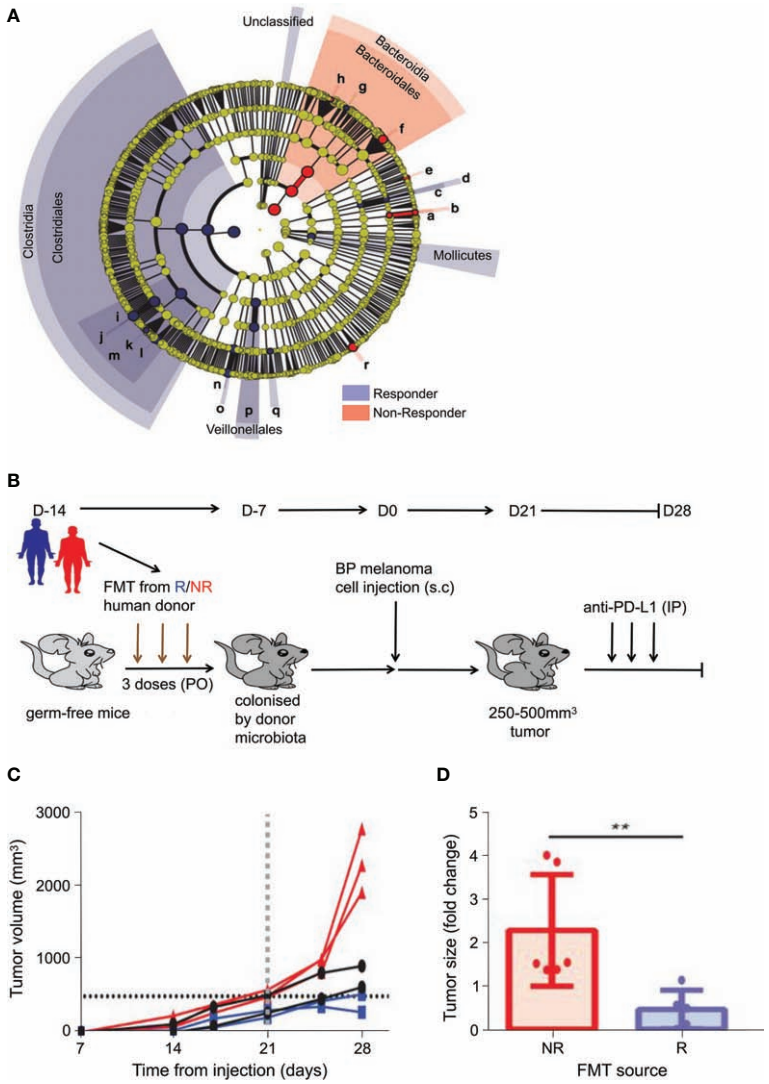

FIGURE 3 | Differences in the composition of intestinal bacteria related to the effects of anti-PD-1 immunotherapy and antitumor immunity. **(A)** Taxonomic cladogram from LEfSe showing differences in stool taxa. The size of dot is positively correlated with the abundance of the taxon. Letters a–r represent the following taxa respectively: (a) *Gardnerella vaginalis*, (b) *Gardnerella*, (c) *Rothia*, (d) *Micrococcaceae*, (e) *Collinsella stercoris*, (f) *Bacteroides mediterraneensis*, (g) *Porphyromonas pasteri*, (h) *Prevotella histicola*, (i) *Faecalibacterium prausnitzii*, (j) *Faecalibacterium*, (k) *Clostridium hungatei*, (l) *Ruminococcus bromii*, (m) *Ruminococcaceae*, (n) *Phascolarctobacterium faecium*, (o) *Phascolarctobacterium*, (p) *Veilonellaceae*, (q) *Peptoniphilus*, (r) *Desulfovbrio alaskensis*. **(B)** Experiment designed to study the GF mice. Relative to days (indicated as D) of tumor injection (2.5 - 8 × 10^5 tumor cells). **(C)** Tumor growth curves for each GF mouse from anti-PD-L1-treated R-FMT (blue, n = 2; median tumor volume = 403.7 mm^3), NR-FMT (red, n = 3; median tumor volume = 2301 mm^3), and Control (black, n = 2; median tumor volume = 771.35 mm^3) mice. Statistics are as follows: p = 0.20 (R-FMT vs NR-FMT), p = 0.33 (NR-FMT vs Control) by the MW test. The black dotted line indicates the size limit of the tumor when treated with anti-PD-L1 (500 mm^3). **(D)** Using the MW test, on the 14th day of implantation in NR-FMT mice (red) and R-FMT (blue), difference in tumor size expressed as fold change (FC) relative to the average tumor volume of Control GF mice. Data from 2 independent FMT experiments (R-FMT, n = 5, median FC = 0.18; NR-FMT, n = 6, median FC = 1.52). ***P* value < 0.01. Adapted from ref. (68).

of acquired immunity. The thymus-dependent antigen (TD-Ag) stimulated B-lymphocytes to produce antibodies, with the participation of not only T- and B-lymphocytes but also accessory cells. In general, APCs include macrophages, DCs, B–lymphocytes, and other cells that can express MHC class II molecules, the so-called full-time APCs. Other cells, such as the fibroblasts, endothelial cells, and various epithelial and mesothelial cells, also exhibit certain antigen presenting functions and are thus called non-full-time APCs. The expression of PD-L1 on DCs or macrophages might affect the efficacy of immunologic checkpoint inhibitors and therefore, in theory, could have the potential to predict the efficacy of drugs (102). We then speculate that intestinal bacteria could influence the efficacy of immunotherapy by regulating APCs.

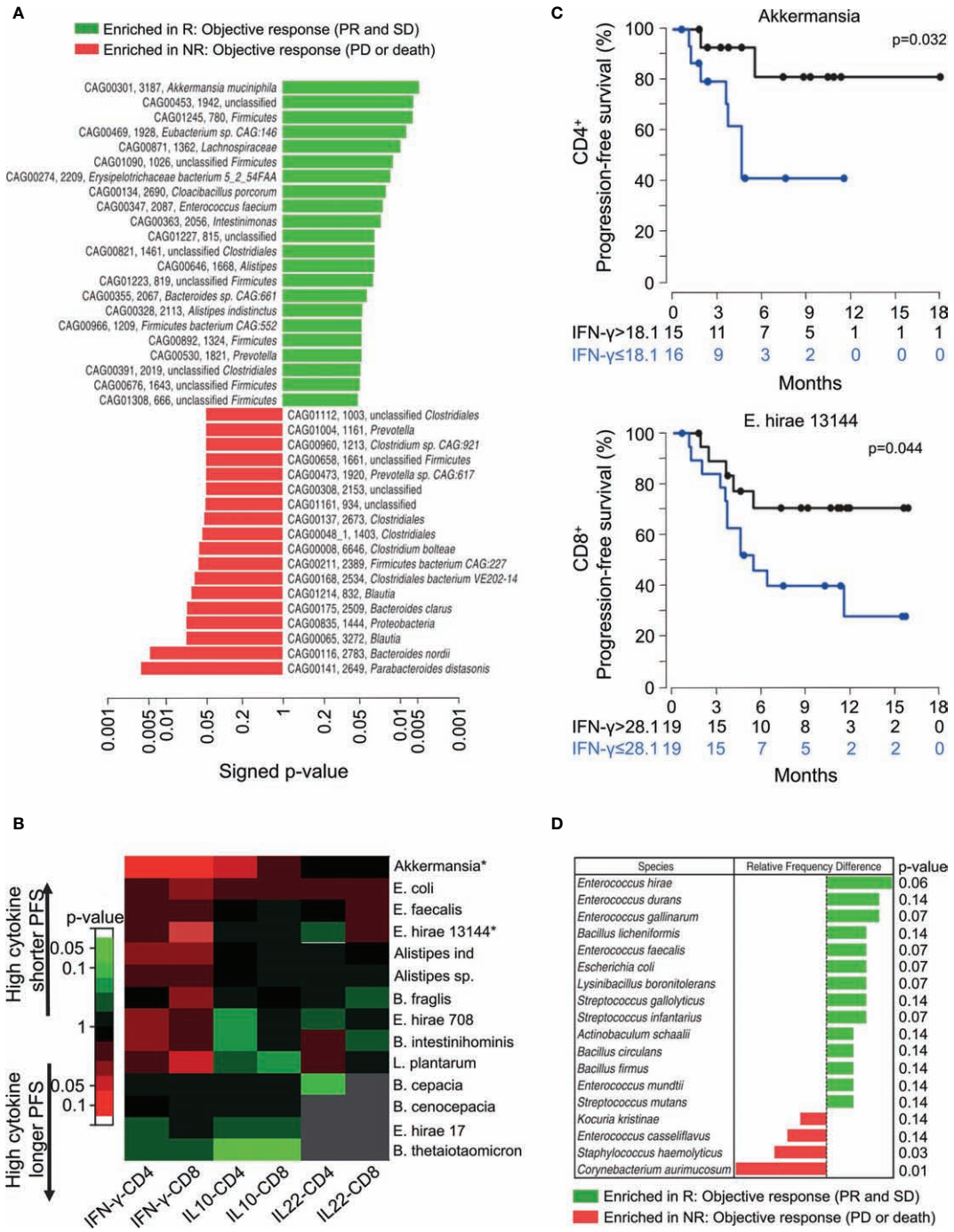

FIGURE 4 | The composition of intestinal bacterial from stool samples determines the efficacy of PD-1 mAb therapy in cancer patients after 3 months. **(A)** Shotgun sequencing of stool samples at the time of diagnosis, using responders (R) (partial response or stable disease) determined according to the best clinical response relative to the non-responder (NR) (progress or death) of each MGS according to RECIST1.1 standard. P value of the entire cohort of n = 100 (60 patients with NSCLC and 40 with RCC). **(B, C)** Immune responses of circulating memory T-cells detected during PD-1 blockade and evaluation of the time to progression. **(B)** Heat map of the P values for each intestinal bacterium and each cytokine, classifying the PFS of patients with NSCLC RCC based on the median value of the production of cytokines in the entire cohort. Significant P values (<0.05, Student's *t* test) are marked by asterisks as relevant intestinal bacteria. **(C)** Kaplan-Meier curves and Univariate analysis showing immune responses of PBS against peripheral blood memory Th1 and Tc1 directed against *A. muciniphila* and *E. hirae* 13144, respectively. **(D)** Stool samples of 16 R and 16 NR patients with NSCLC (defined as the best clinical outcome) analyzed based on culturomic before treatment; each intestinal bacterium having been identified by mass spectrometry. Colored bars show relative frequencies of each commensal in all stool cultures in R over NR patients, and the right graph shows P values with difference. *P value < 0.05. Adapted from ref. (45).

DCs

Discovered by the Canadian scholar Steinman in 1973, DCs are the most powerful antigen-presenting cells at present. They were named for their dendritic or pseudopodal protrusions at their mature state. DCs can absorb, handle, and present antigens efficiently. Immature DCs show a strong ability to migrate, whereas mature DCs can effectively activate primary T-cells, which are essential for initiating, regulating, and maintaining immune responses. DCs have been closely linked with the occurrence and development of tumors. For instance, in the majority of solid tumors, higher DC infiltration correlates with better tumor prognosis. Effective antitumor immune responses mainly relies on the production of cellular immune responses based on $CD8^+$ T-cells, which is also the basis of DCs in immunotherapy.

A previous study showed that C57BL/6 mice raised in Taconic Farms (TAC) or Jackson Laboratory (JAX) animal facilities showed differential colonization by segmented filamentous bacteria (SFB) (103). The difference in growth environment leads to the different composition of intestinal bacteria. Compared with JAX mice, an obvious enrichment of Th17 cells in TAC mice was observed. After 10 days of colonization of JAX-derived GF mice with SFB, the SFB-colonized lamina propria of both the small and large intestines were populated with increased Th17 cells. This suggests that intestinal bacteria, such as SFB, can stimulate immune response *via* direct contact with body tissues. However, further research found that SFB induced the production of serum amyloid A (SAA) by the terminal ileum, with SAA promoting the differentiation of Th17 cells under the action of DCs *in vitro*. The colonization of SFB led to the secretion of SAA, which further stimulated DCs in the intestine to induce the differentiation of Th17 cells, thus demonstrating that intestinal bacteria regulate host immunity through their secretory function.

In addition, substances with immunomodulatory functions, such as IDO, are not only regulated by intestinal bacteria (104, 105) but also expressed in immune cells, which makes the relationship between intestinal bacteria and immune cells more intricate. In fact, there are a series of human APCs that express IDO co-expressing cell surface markers CD123 and CCR6, by which they can be identified from other immune cells (106). In the family of DCs, IDO-expressing DCs can suppress inhibitory effector T cells and promote the differentiation of Tregs, proving to be beneficial in patients with autoimmune diseases like IgA nephropathy (IgAN) (107).

Sivan et al. studied the growth of melanoma in TAC and JAX mice with different commensal intestinal bacteria and observed differences in spontaneous antitumor immunity (15). Particularly, they found that as the most distinct intestinal bacterial species between the two groups, *Bifidobacterium* (**Figure 5A**) unexpectedly enhanced antitumor immunity *in vivo*. *Bifidobacterium* alone or combined with anti-PD-L1 treatment effectively inhibited tumor growth (**Figure 5B**). Additionally, after its oral administration, the antitumor activity of $CD8^+$ T-cells was improved, and it was attributed to DC-induced accumulation of enhanced $CD8^+$ T-cells in the TME. **Figure 5C** displays that the key genes related to antitumor immunity in DCs that are significantly enhanced by the administration of *Bifidobacterium*. Notably, only live *Bifidobacterium* produced this effect, suggesting that *Bifidobacterium* colonized intestinal niches that enabled them to interact with host cells that regulate DCs or to systemically release soluble factors that enhanced the function of DCs. In addition, *Bifidobacterium* was eliminated in $CD8^+$ T-cell-depleted mice, indicating that the regulatory function of *Bifidobacterium* relied on the activity of cytotoxic T-cells. Therefore, the regulation between intestinal bacteria and immune cells is mutual. Furthermore, *Bifidobacterium* can activate other immune regulatory pathways. For instance, *Bifidobacterium* LMG 13195 or their membrane vesicles promoted the differentiation of immature T-cells into $CD25^+$ $Foxp3^+$ Treg cells by acting on DCs and inducing the production of IL-10 *in vitro* (69). *Bifidobacterium* has been extensively studied and can be detected in the intestines after the first meal. It is also a typical probiotic due to its health promoting functions. As a member of the Bifidobacterium family, *Bifidobacterium* LMG 13195 is safe for human consumption. Therefore, *Bifidobacterium* LMG 13195 has the potential as a safe and effective adjuvant for immunotherapy in clinical practice.

Intestinal bacteria were also found to affect the immunotherapy of IBD by regulating DCs. As one of the most potent anti-inflammatory cytokines, IL-10 is required for protection during inflammation. In an experimental colitis model, researchers found that the PSA of *B. fragilis* protected subjects from inflammatory disease *via* promoting the proliferation of IL-10-producing $CD4^+$ T-cells (70). Unlike other polysaccharides, PSA can be internalized by APCs and subsequently presented to T-cells, along with MHC class II molecules. Furthermore, the PSA of *B. fragilis* executed this process by regulating bone-marrow-derived DCs (BMDCs). Therefore, PSA may play a protective role during inflammation. The immunomodulatory capacity of symbiotic factors, such as PSA, may thus provide new treatment approaches for human inflammatory diseases.

In summary, the immunoregulatory role of DCs is multifaceted: DCs can inhibit immunity and exert a beneficial effect on autoimmune diseases such as IgAN, while they can also activate immune responses for immunotherapy of other diseases including tumors. Therefore, intestinal bacterial species that can regulate DCs may have diverse therapeutic applications. However, increasing attention must be paid to the changes in DCs mediated by pathogenic intestinal bacteria.

Macrophages

Macrophages have many functions and are important targets in the study of cell phagocytosis, cellular immunity, and molecular immunology. In vertebrate animals, macrophages are known to be involved in specific (cellular immunity) and nonspecific (innate immunity) defense. Their main function is to engulf pathogens and cellular debris. In addition, macrophages can activate multiple immune cells to release a variety of cytokines (108) and also regulate the differentiation and mobilization of neutrophils *via* secretion of granulocyte-colony stimulating

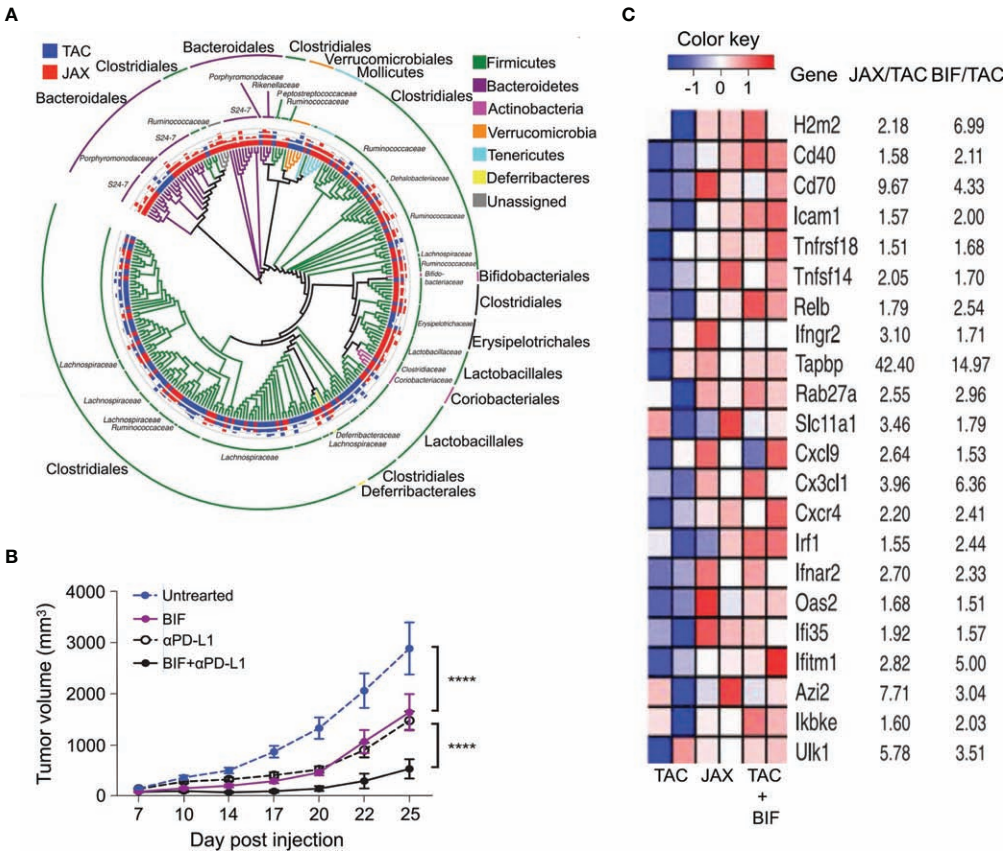

FIGURE 5 | Tumor patients can benefit from direct administration of *Bifidobacterium*, which was shown to improve the DC cell-related tumor-specific immunity and effect of anti-PD-L1 monoclonal antibody treatment. **(A)** In newly obtained TAC and JAX mice, the phylogenetic analysis of taxa with obviously different abundance FDR < 0.05 (nonparametric t test); bars represent log-transformed fold changes, inner circle, log10(10); middle circle, log10(100); outer circle, log10(1000). **(B)** 7 and 14 d after the implantation of B16.SIY tumor, the tumor growth kinetics in TAC mice, untreated or treated with *Bifidobacterium*, anti-PD-L1 mAb 7, 10, 13, and 16 d after tumor implantation, or both regimens. **(C)** Heat map of key antitumor immunity genes in DCs isolated from untreated TAC, *Bifidobacterium*-treated TAC, and JAX mice. Mean fold change for each gene transcript is shown on the right. ****P value < 0.0001. Adapted from ref. (15).

factor (G-CSF) (109). Through these cytokines, macrophages can modulate the activity of other immune cells, such as neutrophils.

As we mentioned before, the PSA of *B. fragilis* provided protection against inflammatory disease *via* the production of the IL-10 cytokine. The relationship between macrophages and IL-10 was further demonstrated by Denning et al. In the lamina propria, some macrophages expressed several anti-inflammatory molecules, including IL-10; however, even after stimulation by Toll-like receptor ligands, the macrophages expressed only low levels of proinflammatory cytokines (110). The differentiation of $Foxp3^+$ Tregs could be induced by these macrophages *via* a mechanism relying on the exogenous transforming growth factor-β, retinoic acid, and IL-10. Although this study did not use intestinal bacteria to regulate the immune process, the regulation of IL-10 by *B. fragilis* or the PSA they produce has been mentioned before. For example, Round and coworkers showed that the transformation of $CD4^+$ T-cells into $Foxp3^+$ Tregs that produce interleukin 10 (IL-10) is mediated by the PSA of *B. fragilis* (43). Therefore, we speculate that the PSA of *B. fragilis* synergizes with macrophage activity to enhance their immunomodulatory functions. In another study, the nucleoprotein fraction (NPF) of *Bacillus subtilis* 7025 cultural medium filtrate stimulated immune responses and exhibited antitumor effects (27). As NPF is mainly composed of histone and protamine, the NPF of *Bacillus subtilis* 7025 cultural medium filtrate has sufficient biological safety and can potentially be used in antitumor immunotherapy as it displays significant immunostimulatory effects through enhanced IFN-γ activity. Moreover, as one of the major cytokines involved in antitumor protection, IFN-γ can activate macrophages.

Remarkably, special macrophages called the microglia have also been reported to be regulated by intestinal bacteria. Microglia, which are found in the brain, are macrophages of the central nervous system (CNS) mainly involved in CNS diseases. Following the stimulation of inflammation, the antigenicity of microglia is enhanced. Host intestinal bacteria have been found to be vital in microglial homeostasis as microglia in GF mice exhibited obvious defects, changes in cell

ratio, and immature phenotypes, resulting in impaired innate immune responses (71). In addition, less complex intestinal bacteria can lead to microglia defects, whereas the reconstitution of their complexity can partially restore the characteristics of microglia. Studies on SCFAs, which are bacterial fermentation products, found that SCFA influences microglial homeostasis and can be pathogenic to the CNS (111).

B-Cells

Progenitor cells of B lymphocytes (B-cells) are found in the island of hematopoietic cells in fetal liver. During development, B-cells are produced and differentiate in the bone marrow. B-cells can differentiate into plasma cells under antigenic stimulation, which can then synthesize and secrete antibodies, mainly involved in humoral immunity.

Intestinal bacterial DNA has been reported to have immunostimulatory effects on B-cells, thereby inducing the production of various cytokines critical in anti-tumor immunity (112). In 1991, researchers found that bacterial DNA could induce significant antibody responses in mice (72). When highly purified ssDNA collected from *E. coli* was used to stimulate lymphocytes, a dose-dependent response was observed, indicating lymphocyte proliferation. As the consumption of T-cells was not observed to reduce the proliferation of lymphocytes, bacterial DNA may have directly triggered the proliferation of B-cells. Another study showed that in bacterial DNA, unmethylated CpG dinucleotides rapidly activate B-cells to secrete IL-6 and IgM (73).

Other Immune Cells

The DNA of intestinal bacteria can activate NK cells and thus enhance antitumor immunity (74, 113). The administration of mycobacterial DNA can induce the secretion of interferon and enhance the activity of NK cells, resulting in tumor regression (114). An *in vitro* experiment showed that the nucleic acid-rich component from Bacille Calmette-Guerinvaccine (BCG) enhanced the activity of NK cells in mouse spleen, induced antiviral activity, and induced macrophages to produce cytotoxic factors against tumor cells.

Group 3 innate lymphoid cells (ILC3) are tissue-resident lymphocytes abundant in mammalian intestine and play an important regulatory role in intestinal inflammation and homeostasis (115). ILC3 proliferation has been associated with *L. paracasei* abundance *(*116*).* This phenomenon was also observed in the lungs after viral infection, subsequently suppressing the inflammatory response to achieve a therapeutic effect *via* mediating the proliferation of Th2 cells (117).

BIOMATERIALS FOR THE DELIVERY OF INTESTINAL BACTERIA

In the previous section, we have introduced the effects of intestinal bacteria on the function of various types of immune cells, which ultimately enhanced immunotherapeutic effect in many diseases. For example, FMT is usually the preferred method to study of intestinal bacteria as a whole. When intestinal bacteria are regulated using FMT, their integrity can be maintained, and the influence of different bacteria on the regulation of the immune function does not need to be considered. In addition, the transfer of fecal samples from experimental mice to GF mice can not only reconstruct the composition of the intestinal bacteria but also facilitate the study of its effect on disease treatment.

However, FMT experiments cannot reveal the mechanisms and the role of certain intestinal bacteria in the regulation of immunotherapy. Therefore, to study the functions of several specific strains, single bacteria or combinations of several probiotics are delivered by oral administration (gavage) (26, 44) or intravenous injection (118). Intestinal bacteria can be delivered directly to the host through intravenous injection, gavage, and even anal perfusion (119, 120); however, these methods have certain disadvantages. Intravenous injection might lead to a variety of infectious diseases, such as bacteremia and septicemia. Especially in bacterial infectious diseases, there is a high risk of bacteria contaminating the blood. Thus, intravenous injection is rarely used in the studies we have discussed here. Anal infusion is more inconvenient than gavage and reduces patient compliance. Besides, intestinal bacteria are widely distributed in the colon. Therefore, the proper delivery of the target bacteria to various parts of the colon to simulate the situation of bacterial distribution in the human body is essential. However, anal perfusion can only deliver the target intestinal bacteria to areas near the descending colon and rectum and not the whole intestine. Thus, oral administration is the most widely used method for the delivery of intestinal bacteria.

Direct oral administration allows the passage of intestinal bacteria through the esophagus, stomach, small intestine, and colon in the proper order. However, this process involves the serious loss in bacterial activity due to the strong acidic environment in the stomach (pH = 2) and bile salts in the intestine (121, 122). This limitation might thus restrict the recognized roles of intestinal bacteria in promoting immunotherapy. Therefore, encapsulating intestinal bacteria with suitable materials conferring them protection and allowing their targeted release in the colon would be an effective method to address this issue.

Due to the strong acidic environment in the stomach, the materials used for bacterial encapsulation should be acid-resistant to maintain the integrity of the microcapsules during their travel to the colon. In addition, these materials should be biocompatible and automatically degraded in the colon to ensure host safety. For these purposes, various biomaterials, including alginate (123), enteric polymer (124), chitosan (125), and pectin (126), have been designed for the encapsulation and effective delivery of intestinal bacteria (122). **Table 2** summarizes these biomaterials.

BIOMATERIALS FOR THE DELIVERY OF *BIFIDOBACTERIUM*

Bifidobacterium is the most abundant bacterial genus in the intestines of breastfed infants (148) and is one of the main

TABLE 2 | Biomaterials used for the oral delivery of intestinal bacteria.

Biomaterials	Bacteria	Encapsulating method	Reference
Calcium alginate/protamine (CAP)	*Lactobacillus casei*	Extrusion	(127)
Microcrystalline cellulose (MCC), calcium cross-linked alginate and lactose	*Lactobacillus casei*	Extrusion Eudragit coating	(128)
Sodium caseinate (SC) Fat sodium caseinate (FSC)	*Lactobacillus casei*	Emulsification	(129)
Alginate Fenugreek Gum Locust Bean Gum	*Pediococcus pentosaceus* KID7 *L. plantarum* KII2 *L. fermentum* KLAB6 *L. helveticus* KII13	Extrusion	(130)
Pea protein isolate–alginate capsules (PPCs)	*Lactobacillus reuteri* ATCC 53608	Extrusion cross-linking	(131)
Alginate–milk microspheres	*Lactobacillus bulgaricus*	Extrusion	(132)
Enteric polymer films Bile adsorbent resins	*Salmonella Typhimurium* SL3261	polymer film laminate (PFL)	(133)
Ethylcellulose Eudragit L100 55	*Bifidobacterium breve* NCIMB 8807	Polymer Film Laminate (PFL)	(124)
Cellulose Calcium carbonate Ca-alginate	*L. Plantarum*	Extrusion	(134)
Alginate-CNC-lecithin microbeads	*Lactobacillus rhamnosus* ATCC 9595	Freeze-drying	(135)
Alginate-silica microcapsules	*Lactobacillus rhamnosus* GG	Electrospraying Mineralization	(136)
Ethylenediaminetetraacetic-calcium-alginate	*Lactobacillus rhamnosus* ATCC 53103	Emulsification	(137)
Liposomes	*Escherichia coli*	Inverse-emulsion	(138)
Alginate-chitosan-alginate (ACA) microcapsules	*Escherichia coli* DH5	Electrostatic interactions	(139)
Cellulose microgels (CMs) Alginate	*Lactobacillus plantarum*	Extrusion	(140)
Pectin-starch hydrogels	*L.plantarum* ATCC 13643	Extrusion	(126)
Corn starch	*L. plantarum* 299v	Freeze-drying	(141)
Alginate Chitosan coating	*L. plantarum*	Extrusion	(142)
Alginate-chitosan	*Bifidobacterium longum*	Surface coating	(125)
Alginate-chitosan	*Bacillus coagulans* (BC)	Electrostatic interactions	(143)
Alginate/poly-llysine/pectin/poly-l-lysine/alginate (APPPA)	*Lactobacillus reuteri*	/	(144)
Pea protein-polysaccharide	*Bifidobacterium*	Extrusion	(145)
Alginate-chitosan	*Bifidobacterium breve*	Surface coating Layer-by-Layer	(146)
Alginate-chitosan	*L. plantarum* PBS067 *L. rhamnosus* PBS070 *Bifidobacterium animalis subsp. lactis* PBS075	Surface coating Emulsion	(147)

components of the gut microbiome associated with the maintenance of human health. The abundance and diversity of *Bifidobacterium* species, including *B. longum*, *B. breve*, and *B. adolescentis*, change throughout life. In the human intestine, *Bifidobacterium* levels decrease with age. In the body of infants and children less than 3 years old, the primary Bifidobacterial species is *B. breve*; in breastfed infants and young adults, the proportion of *B. adolescentis* gradually increases and becomes the main species; in the elderly, *B. longum* becomes the representative species (149, 150). In addition, these *Bifidobacterium* subpopulations exhibit specific health-promoting functions. For example, both *B. infantis* and *B. breve* in the intestine of infants prolonged the immune memory of vaccines (151). *B. longum* in adult intestines promoted immunotherapy response to tumors (24). Because of their excellent health promoting effects and wide applicability, many studies on the microencapsulation of *Bifidobacterium* have been conducted.

Yeung et al. designed a core–shell microgel consisting of an alginate core and a chitosan shell to encapsulate *B. longum* (125). Under aerobic storage and simulated gastric fluid (SGF), the viability and resistance of encapsulated *B. longum* were significantly improved. In 2015, Varankovich et al. developed protein–polysaccharide capsules for *B. adolescentis* (145). Compared with free bacteria, the established capsules provided significant protection to bacterial cells at 37°C in SGF. Moreover, when alginate or iota-carrageenan was used as polysaccharide, the capsules were easily dissolved, releasing 70–79% of bacterial cells into the simulated intestinal fluid (SIF) within 3 h. Moreover, when the capsules were freeze-dried, the number of live bacterial cells released was increased. Another study employed an alginate matrix for *B. breve* followed by alternate encapsulation using alginate and chitosan to coat bacteria using layer-by-layer (LbL) method (146). These multilayer-coated alginate matrices enhanced the viability of *Bifidobacterium breve* in a low-pH environment and delivered bacterial cells

into the intestine, wherein their load was gradually released. Moreover, D'Orazio et al. developed chitosan-coated alginate microcapsules for *B. animalis* subsp. lactis PBS075, *L. rhamnosus* PBS070, and *L. plantarum* PBS067 (147). They found that in SGF and other adverse conditions, the encapsulated probiotics showed significantly higher resistance.

BIOMATERIALS FOR THE DELIVERY OF *LACTOBACILLUS RHAMNOSUS*

L. rhamnosus has been extensively studied since the 1980s. This species has a certain tolerance to gastric acid and bile. By adhering to intestinal cells, it can colonize the human body and has been shown to exert a variety of effects, such as reducing cholesterol levels, inhibiting α-glucosidase activity, and antioxidant and anti-inflammatory effects (152). LGG, a strain of *L. rhamnosus*, can reduce the expression of some inflammation markers and increase the levels of tumor necrosis factor-α, IL-10, and IL-12 in the macrophages to promote a type 1 immune response (153).

Its encapsulation and delivery improved immune regulation. Huq and coworkers developed alginate-cellulose nanocrystals (CNC)-lecithin microbeads for *Lactobacillus rhamnosus* ATCC 9595 (135) and found that CNC-lecithin microbeads had higher compression strength than alginate microbeads alone. Additionally, lecithin reduced the damage to bacterial membranes caused by bile salts in the intestine, thereby protecting probiotics. Another study prepared core-shell alginate–silica microcapsules for LGG (154). After the ionogels were formed using LGG and alginate, a silica coat was applied *via* a mild reaction process. In addition to enhancing the viability of the encapsulated LGG, the mesopores in the silica shell prevented bacterial leakage and allowed the diffusion of nutrient metabolites, thus ensuring bacterial growth within the microcapsules.

Because of considerably low pH levels in the gastrointestinal tract, the development of pH-responsive carriers for the protection and controlled release of bacteria in the stomach and intestine is necessary. Based on this view, an ethylenediaminetetraacetic–calcium–alginate (EDTA–Ca–Alg) system for *L. rhamnosus* ATCC 53103 was prepared using emulsification (137). In the acidic environment of the stomach, the structure of hydrogels remained intact and provided protection for the encapsulated bacteria. However, in neutral pH, as the EDTA completely chelates Ca^{2+}, Ca^{2+} was released from the hydrogel structure, leading to its gradual disintegration, which was kept in a soluble state, thus allowing the release of bacteria.

BIOMATERIALS FOR THE DELIVERY OF *ESCHERICHIA COLI*

Escherichia coli is a gram-negative facultative anaerobe and is one of the most characterized model organisms (155). *E. coli* is not only an extensive intestinal symbiont of vertebrates, but also a multifunctional pathogen. Therefore, using the excellent symbiotic characteristics of *E. coli* and technology, such as genetic engineering or plasmid transfection, *E. coli* can serve as a vector to carry target gene for the stable and efficient expression of specific metabolites, such as monoclonal antibodies (156) and enzymes (118), *in vivo*.

Chowdhuri et al. encapsulated *E. coli* using the inverse-emulsion technique to generate unilamellar vesicles (GUV) and verified the protective effect of liposomes on bacterial viability and activity (138). They demonstrated that *E. coli* encapsulated in liposomes could be protected from degradation by proteases in the stomach by prolonging their dissolution under acidic conditions. In addition, alginate–chitosan–alginate (ACA) microcapsules, which showed strong resistance against enzymatic digestion, were developed for the oral delivery of live bacterial cells for therapy (139). In this study, *E. coli* DH5 were encapsulated in ACA microcapsules and exhibited normal survival and growth during its passage through the stomach and intestine. This is due to the stability of ACA microcapsules in SGF.

BIOMATERIALS FOR THE DELIVERY OF *LACTOBACILLUS PLANTARUM*

Lactobacillus plantarum is a lactic acid bacteria with numerous strains, such as *L. plantarum* 80, *L. plantarum* NCIMB 1193, and *L. plantarum* Hu (157). *L. plantarum* can secrete SCFAs, especially butyric acid and acetic acid, and strengthen the intestinal barrier by enhancing epithelial defense (158). In addition, due to its ability to produce various effective bacteriocins (antimicrobial peptides), *L. plantarum* has been extensively used as a food preservative and antibiotic supplement (159).

Li and co-workers used cellulose microgels (CMs) to encapsulate *L. plantarum* (140). The CMs with their porous structure were shown to have an improved ability of carrying bacteria. Conjugation with alginate contributed to better resistance to acidic conditions and enhancement of the survival of bacteria. In 2017, Dafe et al. synthesized pectin-starch hydrogels to encapsulate *L. plantarum* ATCC:13643 cells using the extrusion method (126). By mixing different concentrations of pectin (2, 1.5, 1, and 0.5% wt) and starch (0, 0.5, 1, and 1.5% wt), hydrogels were divided into 4 groups, named Pectin, Pectin/starch1, Pectin/$starch_2$, and Pectin/$starch_3$, respectively. Compared with unencapsulated bacterial cells, those encapsulated in all ratios of pectin–starch hydrogels were found to be highly resistant to SGF solutions and showed higher survival rate. Chen et al. prepared alginate–poly-L-lysine–alginate (APA) microcapsules for L. plantarum 80 (LP80) (160). However, APA microcapsules only provided short protection in SGF. Upon exposure to an acidic environment (pH = 2) for 5 min, 80.0% of the embedded bacterial cells remained viable. However, after 15 min and 1 h, bacterial viability significantly decreased to 8.3% and 0.2%, respectively. These results represent the limitations of APA microcapsules for the oral delivery of live bacteria. To address these, Nualkaekul et al. designed alginate beads coated with chitosan for

L. plantarum. Their approach enhanced bacterial resistance to both SGF and highly acidic pomegranate juice and increased bacterial survival (142). Furthermore, the multilayer chitosan coating of alginate beads improved their protective function, which increased with the number of coatings.

To prepare sodium alginate/cellulose nanofiber gel macrospheres (ACMs), Zhang et al. extruded a mixture of TEMPO-oxidized cellulose nanofibers (CNF) and sodium alginate (SA) within a CaCl2 solution (**Figure 6A**) (161). To further study the influence of the ratio of SA and CNF on the macrospheres, microspheres were prepared with the ratios of SA and CNF as 1:0, 3:1, 1:1, 1:3, and 0:1, and named them ACM-1, ACM-2, ACM-3, ACM-4, and ACM-5, respectively. After encapsulating the probiotics, *L. plantarum*, ACMs were placed in SGF to simulate the acidic environment in the stomach. After 2 h in SGF, these macrospheres were found to shrink and provide protection for *L. plantarum*, which might be because of the decrease of electrostatic repulsion causing by the protonation of carboxylic chains in CNF and SA (**Figure 6B**). When placed in SIF, *via* deprotonation of carboxylic chains and eliminating intermolecular hydrogen bonds, the ACMs swelled and finally ruptured to release *L. plantarum*, which targeted delivery of probiotics in the intestine (**Figure 6B**). Moreover, among the five different proportions of ACMs, with the proportion of increased CNF, the shrinkage of ACMs decreased in SGF and their rupture delayed in SIF, which demonstrated that CNF improved the stability of ACMs and SA was used for supplying pH-responsive function. **Figure 6C** shows the SEM images of ACM1 to ACM5 after freeze-drying.

In addition to these synthetic materials, some natural substances have also exhibited good loading capacity of bacteria. For instance, naturally occurring cavities and channels can be found in corn starch. After being expanded by enzymatic hydrolysis, natural corn starch can accommodate probiotics. Based on this property, Li and co-workers used fungal α-amylase (FA), pancreatin (P), and pancreatic α-amylase (PA) to partially hydrolyze corn starch and encapsulate *L. plantarum* 299v (141). Furthermore in using different enzymes, corn starch was treated with each enzyme for different times (30 and 120 min), with the materials after treatment being referred to as FA30, FA120, P30, P120, PA30, and PA120, respectively. These differently treated bacteria-containing corn starch samples showed a better acidic resistance, bile salt resistance, and survival rate, as well as exhibited an elevated delivering efficiency compared with free bacteria.

BIOMATERIALS FOR THE DELIVERY OF *LACTOBACILLUS CASEI*

Lactobacillus casei, a gram-positive stain, is one of the most widely studied strains applied as fermentation starter in cultures (162) and probiotics (163). Due to its health-promoting properties, *L. casei* has been extensively researched. It was

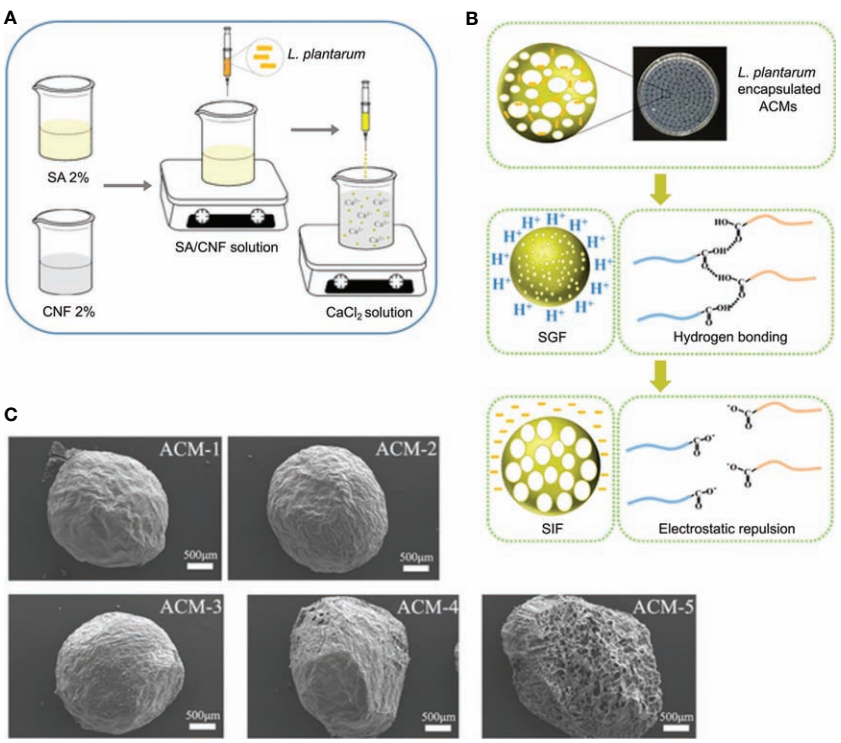

FIGURE 6 | PH-responsive ACMs designed for delivery and release of *L. plantarum*. Overview of **(A)** the process of ACMs embeding *L. plantarum* and **(B)** ACMs designed for protecting *L. plantarum* against SGF and releasing in SIF. **(C)** SEM images of the freeze-dried ACMs. Adapted from ref. (161).

reported to effectively lower blood pressure and cholesterol, enhance human immunity, and inhibit or even prevent tumor growth (164, 165).

To efficiently deliver *L. casei*, Li et al. designed a novel intestinal targeting carrier for the anti-acid protection of *L. casei* and its controlled releasing in the gastrointestinal tract (**Figure 7A**) (127). First, calcium alginate (CA) beads were manufactured using a coextrusion mini-fluidic method of combining pure Ca-alginate solution and Na-alginate solution containing *L. casei*. Then, by employing an adsorption method, the prepared CA beads were adsorbed by protamine molecules to form calcium alginate/protamine (CAP) beads. In addition, sodium caseinate (SC) and lately developed SGF-resistant fat SC (FSC) capsules were also reported to significantly increase the survival of *L. casei* when passing through the upper gastrointestinal tract (129). Although *in vivo* experiments revealed that both SC and FSC capsules were eventually digested in the stomach 3 or 24 h after oral delivery, a high buffer capacity and good emulsification properties were still showed, making them a very suitable encapsulating materials with good research potential. Moreover, de Barro and co-workers produced dried live probiotic spheres (DLPS) of *L. casei* and mixed them with microcrystalline cellulose (MCC), calcium cross-linked alginate, and lactose followed by granulation (128). Subsequently, a Eudragit coating was added using an extrusion method to protect bacteria from acid and

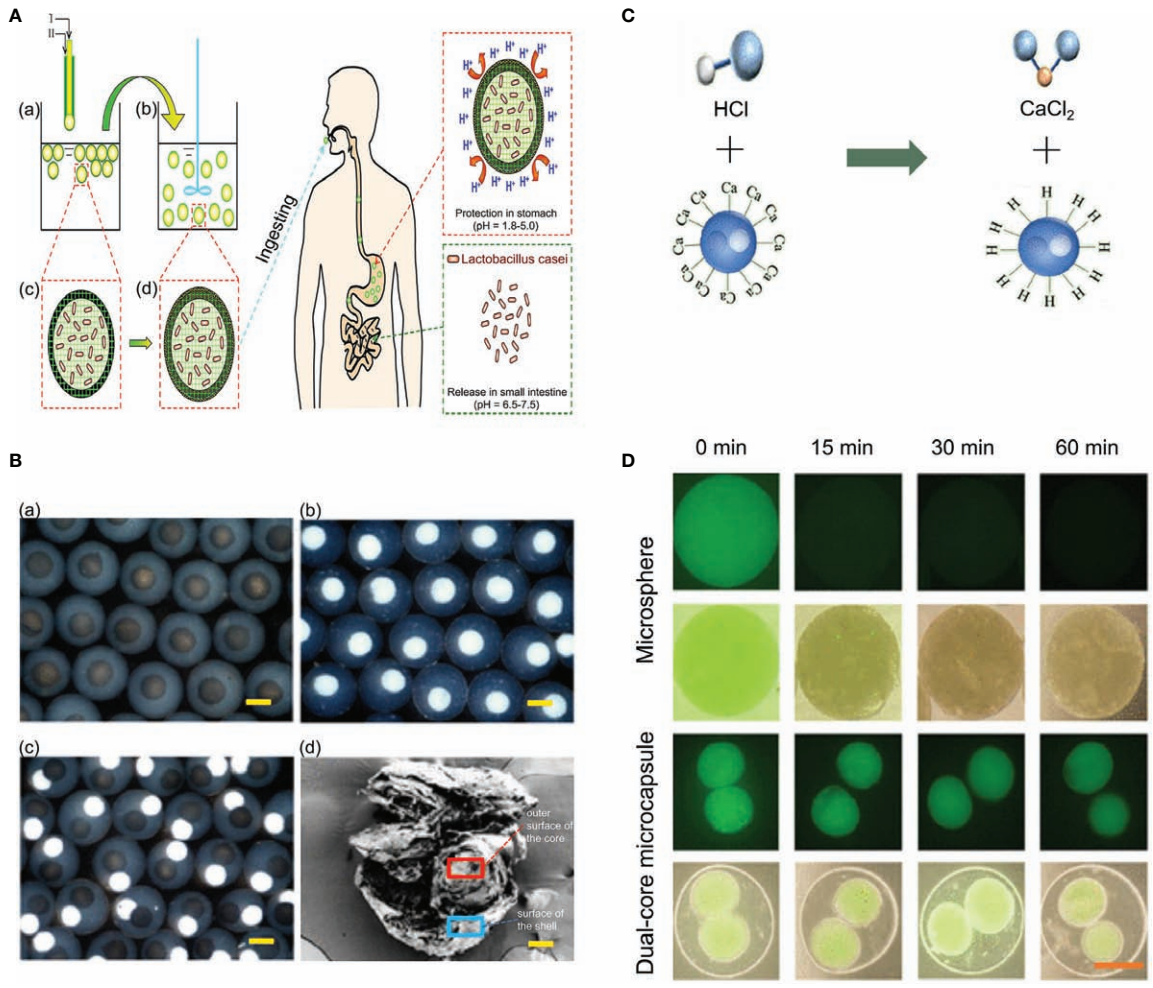

FIGURE 7 | CAP beads and dual-core microcapsules prepared for encapsulating *Lactobacillus casei*, *Bacillus subtilis*, and *Lactobacillus* (NO. 21790). **(A)** Schematic illustration of the preparation process and the design concept of the proposed intestinal-targeted CAP carrier for the pH-responsive protection and release of *L. casei*. (a, c) CA beads prepared by a coextrusion method. "A" is Na-alginate solution containing *L. casei*, whereas "B" is pure Na-alginate solution. (b, d) CAP beads prepared by adsorption of protamine molecules. (e) Ingestion of CAP beads. (f) CAP beads offer improved protection to *Lactobacillus* in the stomach. (g) CAP beads rapidly release *L. casei* in the small intestine. **(B)** Characterization of microcapsules. optical microscope images of (a) the *Lactobacillus* microcapsules, (b) the Bacillus Subtilis microcapsules, and (c) dual-core microcapsules. (d) SEM images of dual-core microcapsules. Scale bars are 100 μm. **(C)** Alginate neutralizes HCL through metathesis reaction. **(D)** Detection of the activity of bacteria embeded in microspheres and dual-core microcapsules in SGF *via* fluorescent staining. The scale bar is 100 μm. Adapted from ref. (127, 166).

maintain the integrity of the entire structure in the acidic environment of the stomach. When reaching the intestine, increase in the pH led to the change of the Eudragit coating from hydrophobic to hydrophilic and the concomitant release of the bacterial cells.

BIOMATERIALS FOR THE DELIVERY OF OTHER BACTERIA

In addition to the intestinal bacteria mentioned above, there are also many other bacteria in the intestine that are known to have the function of promoting the health of the human body. To make the oral delivery of these intestinal bacteria more efficient, many suitable biomaterial schemes have been designed for their encapsulation.

Based on the yin–yang concept, microcapsules with independent internal compartments, which can encapsulate and deliver a variety of substances, such as drugs and microbes, to promote their functions without interference, have been designed (167, 168). Zhao and coworkers designed a dual-core microcapsule to encapsulate and deliver *B. subtilis* and *Lactobacillus* (NO. 21790) in separated microcompartments (166). First, each intestinal bacterium was mixed in a solution containing CNC, carboxymethyl cellulose (CMC), and sodium alginate. Then, it was electrosprayed to prepare the microspheres (containing only one kind of intestinal bacteria). In this technique, CMC had the function of neutralizing HCl to protect bacteria in an acidic environment, while CNC could restrict these bacteria to a certain range *via* depletion flocculation. Aluminum chloride ($AlCl_3$) was the chosen crosslinking agent as it caused the microspheres that encapsulated the intestinal bacteria to form quickly. Subsequently, the outer phase fluid could be wrapped around the inner core by hydrodynamic focusing, and the alginate could be quickly solidified after the solution containing the dual cores was placed in the presence of electric field in a gel bath with 2% CaCl2, thereby forming the dual-core microcapsules. **Figure 7B** shows the optical microscope and SEM images of dual-core microcapsules. To further study the protective effect of dual-core microcapsules on bacteria in an acidic environment, dual-core microcapsules and microspheres without alginate shells were placed in simulated gastric juice (SGF) for 60 min to observe the bacterial activity. Theoretically, the dual-core microcapsules could provide protection to the encapsulated bacteria in an acidic environment because of the sodium alginate shell, which could neutralize HCL through a metathesis reaction (**Figure 7C**). As shown in **Figure 7D**, compared with dual-core microcapsules, intestinal bacteria encapsulated in microspheres without alginate shells lost most of their activity in the first 15 min. However even after 60 min, more than 70% of the probiotics encapsulated in dual-core microcapsules retained their activity in SGF.

Anselmo et al. combined chitosan (CHI) with alginate (ALG) as one CHI/ALG bilayer and then encapsulated intestinal bacteria using LbL method (**Figure 8A**) (143). In their study, *Bacillus coagulans* was used as a model strain. Several different LbL formulations and numbers of layer were designed to research their protecting function. An enteric polymer, L 100, was chosen to encapsulate probiotics in combination with chitosan. Compared with *B. coagulans* encapsulated using chitosan only, L100 combined with chitosan (denoted as CHI/L100) was able to protect the probiotic in SGF but failed against bile (**Figure 8B**). *B. coagulans* encapsulated by 2 CHI/ALG bilayers, expressed as $(CHI/ALG)_2$, was reported to be well protected against both SGF and bile (**Figure 8B**). **Figure 8C** shows that bacteria encapsulated by $(CHI/ALG)_2$ presented higher adhesion to the intestine in slices of freshly isolated small intestine. Additionally, the $(CHI/ALG)_2$ coating was shown to improve the survival of probiotics in the intestine. Ouyang and co-workers improved the APA microcapsules and designed multilayer alginate/poly-L-lysine/pectin/poly-L-lysine/alginate (APPPA) microcapsules to encapsulate *L. reuteri* cells (144). Stability of APPPA microcapsules were tested as well as the activity of encapsulated bacterial cells at 37.2°C under various pH conditions. When microcapsules were placed into SGF and SIF, respectively, for a total of 24 h (12 h in SGF and 12 h in SIF) at 250 rpm mechanical shaking at 37.2°C, no obvious damage to bacterial cells was reported. After 24 h in GSF and SIF, respectively, more than 90% of APPPA microcapsules remained intact, exhibiting a good resistance to mechanical shocks. Compared with APA microcapsules, APPPA microcapsules showed an excellent stability. The reason behind this was the use of poly (amino acid), which is a material that has been widely used for encapsulating bacteria. It was therefore assumed that using more poly-L-lysine to encapsulate bacteria would have a better protection and delivery effect. Besides, when designing biomaterial for encapsulating bacteria, the number of layers is also considered to be an important factor affecting delivery efficiency. Accordingly, the effect of this protection was proportional to the number of layers in a certain range, rather than with the increasing in the number of layers (146).

BIOMATERIALS FOR DELIVERING BACTERIAL DNA

Many intestinal bacteria can regulate the immune function and promote host health mainly *via* their DNA (169). Therefore, the encapsulation and delivery of bacterial DNA has great potential. As a positively charged natural polymer, chitosan can entrap nucleic acids (NA) (both RNA and DNA) and protect them from degradation by nuclease (170). However, chitosan is rarely used as NA carrier because of its poor water solubility and low transfection efficiency. To overcome these limitations, various modifications and formulations have been proposed (171, 172). For instance, Zhang et al. developed the PEGylation of chitosan nanoparticles for the delivery of DNA. These chitosan–DNA–PEG complexes increased the dose of DNA delivered and have been indicated to exhibit a liver tumor targeting (173). In another study, chitosan salts, such as chitosan glutamate, chitosan aspartate, chitosan acetate, chitosan lactate, and chitosan hydrochloride, were used to form chitosan–DNA complexes (174). Compared with standard

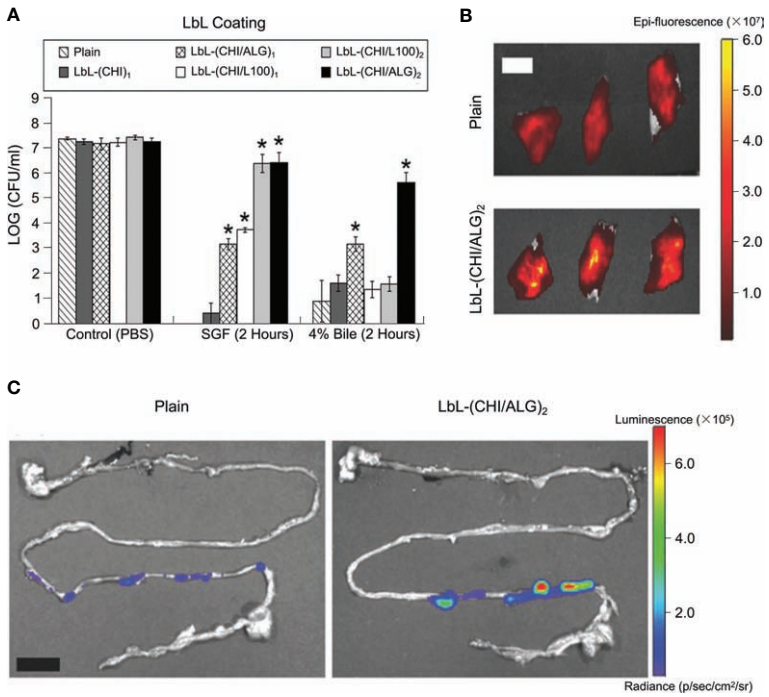

FIGURE 8 | Layer-by-layer encapsulation of probiotics employed to enhance their survival rate against acidic and bile conditions, and their physical retention in the intestine. **(A)** Schematic LbL encapsulation of chitosan and alginate on probiotic. **(B)** LbL formulated $(CHI/ALG)_2$ (black bars) BC were protected against both acidic and bile salt conditions at 37°C for up to 2 h. LbL coatings of chitosan (dark gray bars), $(CHI/L100)_1$ (white bars), $(CHI/L100)_2$ (light gray bars), and $(CHI/ALG)_1$ (cross-hatched bars) were less effective at protecting BC against both acidic and bile conditions. Error bars represent standard deviation (n = 3). *denotes statistical difference ($P < 0.05$) using Student's *t*-test between plain and LbL groups. **denotes statistical difference ($P < 0.05$) using individual Student's *t*-test between the designated and any other group. **(C)** IVIS images of porcine intestine with plain- and $(CHI/ALG)_2$-probiotics. Adapted from ref. (143).

chitosan, these complexes showed better transfection efficiency and lower cytotoxicity.

CONCLUSION AND PROSPECTS

The regulation of intestinal bacteria to enrich target probiotics might be an effective strategy to improve the immunotherapeutic response. However, the best way to achieve this goal remains to be determined. When intestinal bacteria are transplanted as a whole (such as in FMT), thousands of bacterial species are simultaneously introduced. As such, this approach is not conducive to the in-depth study of the functions of each bacterial species. In addition, although the combination of intestinal bacteria in FMT has been shown to be of vital importance in this treatment, FMT can affect the abundance of main bacteria, while other bacteria do not exhibit any effect or even reduce the function of the main probiotic. Even if we could isolate the main bacteria, the immunomodulatory effects of the same strain would not appear to be completely consistent in different diseases and different studies. As we mentioned earlier, *Lactobacilli* populations showed diametrically opposite regulatory functions for Tregs in different studies. Therefore, we considered that the regulation of immunotherapy by intestinal bacteria could be affected by other factors.

Early in 2006, Hara et al. considered intestinal bacteria as a whole and proposed them as a potential organ of the human body (175). When artificially enriching or eliminating the number of intestinal bacterial species, this is generally accompanied by the reestablishment of a new multibacterial environment. This significant change in the composition of intestinal bacteria might explain the significant differences observed in the role of certain bacteria and might explain the positive effects of regulating intestinal bacteria on the efficacy of disease immunotherapy. In addition, intestinal bacteria are also known to be interdependent with the immune cells that they regulate. For example, *Bifidobacterium* was reported to improve the effect of PD-L1 inhibitor in antitumor treatment by regulating cytotoxic T-cells (15). However, *Bifidobacterium* could not exhibit this immunomodulatory effect in cytotoxic T-cell-depleted mice, suggesting that its effect was depended on the activity of cytotoxic T-cells. More specifically, it was assumed that intestinal bacteria serve more as aggregators or amplifiers of immune cells, which can in turn enhance the efficacy of immunotherapy. However, this regulatory function was demonstrated to rely on the adequacy of immune cells and the integrity of the immune system. Besides their interaction with

immune cells, intestinal bacteria have also been shown to avoid or alleviate pathological conditions in various diseases, such as nonspecific inflammation. For example, $Foxp3^+$ Tregs were found to be widely involved in the immunotherapy of various diseases. This was attributed to the role of $Foxp3^+$ Tregs in affecting immune responses and suppressing the progress of inflammation. Based on this feature, several probiotics and the PSA of *B. fragilis* have been used to inhibit the progression of inflammation through the upregulation of $Foxp3^+$ Tregs and to promote the immunotherapy of IBD (70) or the HIV-1 infection (67). Therefore, focusing on these common beneficial effects of intestinal bacteria and trying to combine them in treatment applications against various diseases or clinical problems might greatly expand the application prospects of intestinal bacteria.

The regulation of immune cells by intestinal bacteria is known to be accompanied by other substances in the microenvironment, cytokines, which can recruit and activate different kinds of immune cells. Many of the studies mentioned above have shown that the levels of interleukins change following the administration of intestinal bacteria. Independent of being upregulated or downregulated, the correlation between intestinal bacteria and interleukins has revealed the mechanism of the immune regulation by intestinal bacterial to a certain extent, and has also shown the feasibility of the combined use of these 2 factors for the immunotherapy of various diseases. Apart from interleukins, the levels of chemokines (75) and interferons (15) in the body have also been shown to be affected by intestinal bacteria. In particular, upregulation of IFN-γ by intestinal bacteria (such as *Bifidobacterium*) has also revealed to a certain extent the reason behind the ability of intestinal bacteria to promote the effect of ICB therapy in tumor immunotherapy. Recent studies have found that IFN-γ could upregulate the production of PD-L1 and establish the IFN-γ/PD-L1 axis based on the relationship between them (176–178). Furthermore, IFN-γ was found to exhibit the same effect on the expression of CTLA-4 (179, 180). Respectively, administration of intestinal bacteria to increase the expression of PD-L1 and CTLA-4, would undoubtedly greatly improve the effect of anti-CTLA-4 and anti-PD-1/PD-L1 therapies. Therefore, selecting the appropriate cytokines to synergize with intestinal bacteria could greatly enhance the effect of immunotherapy and the regulatory function of intestinal bacteria. Not only limited to cytokines, this synergy with intestinal bacteria established by immune cells could lead to more research ideas.

Regarding the intestinal bacteria used to regulate immunotherapy, there are also some problems that need in-depth study. One concern is the duration of their regulation of the immune system. As we know, intestinal bacteria are specific to people living in different areas or enjoying different diet habits, similar to our physical characteristics, and this specificity is known to determine the sensitivity of everybody to diseases and immunotherapy. As mentioned earlier, because of the differences in the composition of intestinal bacteria, patients who suffered from melanoma and were treated with PD-1 inhibitor were divided into responders (R) and non-responders (NR). This phenomenon implied that as long as the composition of intestinal bacteria remains stable in the body, their regulatory function might be long-term. Based on this view, intestinal bacteria could not only be used to regulate immunotherapy but might also be a major factor to prevent the diseases. Unlike conventional vaccines or vaccines based on genetically engineered bacteria, this kind of disease prevention *via* the direct use of intestinal bacteria might exhibit better compliance and longer-term preventive effects. Moreover, with deeper studies on their ability for immune regulation, it might be entirely possible to select the most suitable multi-bacterial environment and reconstruct it in unaffected or affected hosts by introducing various probiotics in order to achieve the prevention of many diseases and the maintenance of health status.

However, we would also need to consider how this multi-bacterial commensalism can be established and made stable in the body. First of all, in order to improve compliance and avoid infections caused by bacteria entering the blood, oral delivery of intestinal bacteria should be routinely selected. Second, in order to ensure the vitality of extraneous intestinal bacteria, these should be delivered in the form of microcapsules, that is, bacteria should be encapsulated into microspheres. Therefore, related encapsulation materials and technologies have to be developed to ensure the protection of bacteria from the acidic environment in the stomach and their complete release in the intestine. Furthermore, following the introduction of encapsulated bacteria in the body and the establishment of a new multi-bacterial environment, it would be necessary to maintain this multi-bacterial environment in the long term. As we all know, diet is an important factor in the composition of the intestinal bacteria. Therefore, providing an appropriate diet is an important method to this end. However, its precondition is the knowledge of the relationship between diet and intestinal bacteria. Hence, immunotherapy for many diseases, including tumors, is expected to open a new chapter through the dietary regulation of intestinal bacteria to achieve the prevention of diseases, promotion of immunotherapy during diseases, and maintenance of the curative effect after the resolution of diseases.

In this review, we have outlined the potential use of intestinal bacteria for the regulation of immunotherapy of diseases and the useful biomaterials employed for encapsulating these bacteria. Future studies should focus on developing efficient encapsulation and delivery methods and accurate targeting ability and establishing stable multi-bacterial environments to expand the clinical applicability of intestinal bacteria in disease treatment. In addition, dietary factors and genetic engineering can provide more methods and possibilities for their clinical application. We believe that with the continuous in-depth studies of intestinal bacteria and exploration of their synergistic strategies, better, safer, and more effective intestinal bacterial therapies will be used in clinical practice with more people benefiting from them.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

REFERENCES

1. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PloS Biol* (2016) 14(8):e1002533. doi: 10.1371/journal.pbio.1002533
2. West NR, Powrie F. Immunotherapy Not Working? Check Your Microbiota. *Cancer Cell* (2015) 28(6):687–9. doi: 10.1016/j.ccell.2015.11.010
3. Hold GL. Gastrointestinal Microbiota and Colon Cancer. *Dig Dis* (2016) 34 (3):244–50. doi: 10.1159/000443358
4. Khosravi A, Yanez A, Price JG, Chow A, Merad M, Goodridge HS, et al. Gut microbiota promote hematopoiesis to control bacterial infection. *Cell Host Microbe* (2014) 15(3):374–81. doi: 10.1016/j.chom.2014.02.006
5. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med* (2014) 20(2):159–66. doi: 10.1038/nm.3444
6. De Santis S, Cavalcanti E, Mastronardi M, Jirillo E, Chieppa M. Nutritional Keys for Intestinal Barrier Modulation. *Front Immunol* (2015) 6:612. doi: 10.3389/fimmu.2015.00612
7. Vaishnava S, Behrendt CL, Ismail AS, Eckmann L, Hooper LV. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci USA* (2008) 105(52):20858–63. doi: 10.1073/pnas.0808723105
8. Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol* (2014) 14(3):141–53. doi: 10.1038/nri3608
9. Roy S, Trinchieri G. Microbiota: a key orchestrator of cancer therapy. *Nat Rev Cancer* (2017) 17(5):271–85. doi: 10.1038/nrc.2017.13
10. Dzutsev A, Goldszmid RS, Viaud S, Zitvogel L, Trinchieri G. The role of the microbiota in inflammation, carcinogenesis, and cancer therapy. *Eur J Immunol* (2015) 45(1):17–31. doi: 10.1002/eji.201444972
11. Pitt JM, Vetizou M, Waldschmitt N, Kroemer G, Chamaillard M, Boneca IG, et al. Fine-Tuning Cancer Immunotherapy: Optimizing the Gut Microbiome. *Cancer Res* (2016) 76(16):4602–7. doi: 10.1158/0008-5472.CAN-16-0448
12. Chow J, Tang H, Mazmanian SK. Pathobionts of the gastrointestinal microbiota and inflammatory disease. *Curr Opin Immunol* (2011) 23 (4):473–80. doi: 10.1016/j.coi.2011.07.010
13. Vieira SM, Hiltensperger M, Kumar V, Zegarra-Ruiz D, Dehner C, Khan N, et al. Translocation of a gut pathobiont drives autoimmunity in mice and humans. *Science* (2018) 359(6380):1156–60. doi: 10.1126/science.aar7201
14. Jobin C. Precision medicine using microbiota. *Science* (2018) 359(6371):32–4. doi: 10.1126/science.aar2946
15. Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, et al. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science* (2015) 350(6264):1084–9. doi: 10.1126/science.aac4255
16. Li J, Sung CYJ, Lee N, Ni Y, Pihlajamäki J, Panagiotou G, et al. Probiotics modulated gut microbiota suppresses hepatocellular carcinoma growth in mice. *Proc Natl Acad Sci* (2016) 113(9):E1306–15. doi: 10.1073/pnas.1518189113
17. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* (2011) 472(7341):57–U82. doi: 10.1038/nature09922
18. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci USA* (2011) 108(38):16050–5. doi: 10.1073/pnas.1102999108
19. Zhan Y, Chen PJ, Sadler WD, Wang F, Poe S, Nunez G, et al. Gut microbiota protects against gastrointestinal tumorigenesis caused by epithelial injury. *Cancer Res* (2013) 73(24):7199–210. doi: 10.1158/0008-5472.CAN-13-0827
20. Belkaid Y, Naik S. Compartmentalized and systemic control of tissue immunity by commensals. *Nat Immunol* (2013) 14(7):646–53. doi: 10.1038/ni.2604
21. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* (2013) 504(7480):451–5. doi: 10.1038/nature12726
22. Yu YA, Shabahang S, Timiryasova TM, Zhang Q, Beltz R, Gentschev I, et al. Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins. *Nat Biotechnol* (2004) 22 (3):313–20. doi: 10.1038/nbt937
23. Chua KJ, Kwok WC, Aggarwal N, Sun T, Chang MW. Designer probiotics for the prevention and treatment of human diseases. *Curr Opin Chem Biol* (2017) 40:8–16. doi: 10.1016/j.cbpa.2017.04.011
24. Matson V, Fessler J, Bao R, Chongsuwat T, Zha Y, Alegre M-L, et al. The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. *Science* (2018) 359(6371):104–+. doi: 10.1126/science.aao3290
25. Routy B, Gopalakrishnan V, Daillere R, Zitvogel L, Wargo JA, Kroemer G. The gut microbiota influences anticancer immunosurveillance and general health. *Nat Rev Clin Oncol* (2018) 15(6):382–96. doi: 10.1038/s41571-018-0006-2
26. Ciorba MA, Riehl TE, Rao MS, Moon C, Ee X, Nava GM, et al. Lactobacillus probiotic protects intestinal epithelium from radiation injury in a TLR-2/cyclo-oxygenase-2-dependent manner. *Gut* (2012) 61(6):829–38. doi: 10.1136/gutjnl-2011-300367
27. Shlyakhovenko VA, Olishevsky SV, Kozak VV, Yanish YV, Rybalko SL. Anticancer and immunostimulatory effects of nucleoprotein fraction of Bacillus subtilis 7025 culture medium filtrate. *Exp Oncol* (2003) 25 (2):119–23.
28. Round JL, Lee SM, Li J, Tran G, Jabri B, Chatila TA, et al. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* (2011) 332(6032):974–7. doi: 10.1126/science.1206095
29. Cong Y, Feng T, Fujihashi K, Schoeb TR, Elson CO, dominant A. coordinated T regulatory cell-IgA response to the intestinal microbiota. *Proc Natl Acad Sci USA* (2009) 106(46):19256–61. doi: 10.1073/pnas.0812681106
30. Robert S, Gysemans C, Takiishi T, Korf H, Spagnuolo I, Sebastiani G, et al. Oral Delivery of Glutamic Acid Decarboxylase (GAD)-65 and IL10 by Lactococcus lactis Reverses Diabetes in Recent-Onset NOD Mice. *Diabetes* (2014) 63(8):2876–87. doi: 10.2337/db13-1236
31. Grierson P, Lim KH, Amin M. Immunotherapy in gastrointestinal cancers. *J Gastrointest Oncol* (2017) 8(3):474–84. doi: 10.21037/jgo.2017.05.01
32. Wei P, Yang Y, Liu Z, Huang J, Gong Y, Sun H. Oral Bifidobacterium longum expressing alpha-melanocyte-stimulating hormone to fight experimental colitis. *Drug Delivery* (2016) 23(6):2058–64. doi: 10.3109/10717544.2015.1122672
33. Ly NP, Litonjua A, Gold DR, Celedon JC. Gut microbiota, probiotics, and vitamin D: interrelated exposures influencing allergy, asthma, and obesity? *J Allergy Clin Immunol* (2011) 127(5):1087–94. doi: 10.1016/j.jaci.2011.02.015
34. Blumberg R, Powrie F. Microbiota, disease, and back to health: a metastable journey. *Sci Transl Med* (2012) 4(137):137rv7. doi: 10.1126/scitranslmed.3004184
35. Lucas C, Barnich N, Hang Thi Thu N. Microbiota, Inflammation and Colorectal Cancer. *Int J Mol Sci* (2017) 18(6):1310. doi: 10.3390/ijms18061310
36. Gopalakrishnan V, Helmink BA, Spencer CN, Reuben A, Wargo JA. The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy. *Cancer Cell* (2018) 33(4):570–80. doi: 10.1016/j.ccell.2018.03.015
37. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell* (2014) 157(1):121–41. doi: 10.1016/j.cell.2014.03.011
38. Zitvogel L, Ayyoub M, Routy B, Kroemer G. Microbiome and Anticancer Immunosurveillance. *Cell* (2016) 165(2):276–87. doi: 10.1016/j.cell.2016.03.001
39. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. *Nature* (2011) 480(7378):480–9. doi: 10.1038/nature10673
40. Araya RE, Goldszmid RS. Two Bugs a NOD Away from Improving Cancer Therapy Efficacy. *Immunity* (2016) 45(4):714–6. doi: 10.1016/j.immuni.2016.10.007
41. York A. Microbiome: Gut microbiota sways response to cancer immunotherapy. *Nat Rev Microbiol* (2018) 16(3):121. doi: 10.1038/nrmicro.2018.12
42. Brown CT, Davis-Richardson AG, Giongo A, Gano KA, Crabb DB, Mukherjee N, et al. Gut microbiome metagenomics analysis suggests a

functional model for the development of autoimmunity for type 1 diabetes. *PloS One* (2011) 6(10):e25792. doi: 10.1371/journal.pone.0025792
43. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci USA* (2010) 107(27):12204–9. doi: 10.1073/pnas.0909122107
44. Vetizou M, Pitt JM, Daillere R, Lepage P, Waldschmitt N, Flament C, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* (2015) 350(6264):1079–+. doi: 10.1126/science.aad1329
45. Routy B, Le Chatelier E, Derosa L, Duong CPM, Alou MT, Daillere R, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* (2018) 359(6371):91–+. doi: 10.1126/science.aan3706
46. Ansari F, Pourjafar H, Jodat V, Sahebi J, Ataei A. Effect of Eudragit S100 nanoparticles and alginate chitosan encapsulation on the viability of Lactobacillus acidophilus and Lactobacillus rhamnosus. *AMB Express* (2017) 7(1):144. doi: 10.1186/s13568-017-0442-x
47. Singh P, Medronho B, Valente AJM, Miguel MG, Lindman B. Exploring the prebiotic effect of cyclodextrins on probiotic bacteria entrapped in carboxymetyl cellulose-chitosan particles. *Colloids Surf B Biointerf* (2018) 168:156–62. doi: 10.1016/j.colsurfb.2017.12.014
48. Alkotaini B, Tinucci SL, Robertson SJ, Hasan K, Minteer SD, Grattieri M. Alginate-Encapsulated Bacteria for the Treatment of Hypersaline Solutions in Microbial Fuel Cells. *Chembiochem* (2018) 19(11):1162–69. doi: 10.1002/cbic.201800142
49. Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. *Nat Rev Genet* (2012) 13(4):260–70. doi: 10.1038/nrg3182
50. Prakash S, Jones ML. Artificial Cell Therapy: New Strategies for the Therapeutic Delivery of Live Bacteria. *J BioMed Biotechnol* (2005) 2005 (1):44–56. doi: 10.1155/JBB.2005.44
51. Hanahan D, Coussens LM. Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment. *Cancer Cell* (2012) 21(3):309–22. doi: 10.1016/j.ccr.2012.02.022
52. Pitt JM, Marabelle A, Eggermont A, Soria JC, Kroemer G, Zitvogel L. Targeting the tumor microenvironment: removing obstruction to anticancer immune responses and immunotherapy. *Ann Oncol* (2016) 27(8):1482–92. doi: 10.1093/annonc/mdw168
53. Gajewski TF, Schreiber H, Fu Y-X. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol* (2013) 14(10):1014–22. doi: 10.1038/ni.2703
54. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* (2013) 39(4):782–95. doi: 10.1016/j.immuni.2013.10.003
55. Kroemer G, Zitvogel L. Cancer immunotherapy in 2017: The breakthrough of the microbiota. *Nat Rev Immunol* (2018) 18(2):87–8. doi: 10.1038/nri.2018.4
56. Roesch LF, Lorca GL, Casella G, Giongo A, Naranjo A, Pionzio AM, et al. Culture-independent identification of gut bacteria correlated with the onset of diabetes in a rat model. *ISME J* (2009) 3(5):536–48. doi: 10.1038/ismej.2009.5
57. Chaput N, Lepage P, Coutzac C, Soularue E, Le Roux K, Monot C, et al. Baseline gut microbiota predicts clinical response and colitis in metastatic melanoma patients treated with ipilimumab. *Ann Oncol* (2017) 28(6):1368–79. doi: 10.1093/annonc/mdx108
58. Chen W, Liu F, Ling Z, Tong X, Xiang C. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PloS One* (2012) 7(6):e39743. doi: 10.1371/journal.pone.0039743
59. Wang T, Cai G, Qiu Y, Fei N, Zhang M, Pang X, et al. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *Isme J* (2012) 6(2):320–9. doi: 10.1038/ismej.2011.109
60. Wei H, Dong L, Wang T, Zhang M, Hua W, Zhang C, et al. Structural shifts of gut microbiota as surrogate endpoints for monitoring host health changes induced by carcinogen exposure. *FEMS Microbiol Ecol* (2010) 73(3):577–86. doi: 10.1111/j.1574-6941.2010.00924.x
61. Marchesi JR, Dutilh BE, Hall N, Peters WHM, Roelofs R, Boleij A, et al. Towards the Human Colorectal Cancer Microbiome. *PloS One* (2011) 6(5):e20447. doi: 10.1371/journal.pone.0020447
62. Kriegel MA, Sefik E, Hill JA, Wu HJ, Benoist C, Mathis D. Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice—-65. *Proc Natl Acad Sci USA* (2011) 108(28):11548–53. doi: 10.1073/pnas.1108924108
63. Lau K, Benitez P, Ardissone A, Wilson TD, Collins EL, Lorca G, et al. 3rd, Inhibition of type 1 diabetes correlated to a Lactobacillus johnsonii N6.2-mediated Th17 bias. *J Immunol* (2011) 186(6):3538–46. doi: 10.4049/jimmunol.1001864
64. Cunningham-Rundles S, Ahrne S, Johann-Liang R, Abuav R, Dunn-Navarra AM, Grassey C, et al. Effect of probiotic bacteria on microbial host defense, growth, and immune function in human immunodeficiency virus type-1 infection. *Nutrients* (2011) 3(12):1042–70. doi: 10.3390/nu3121042
65. Dubin K, Callahan MK, Ren B, Khanin R, Viale A, Ling L, et al. Intestinal microbiome analyses identify melanoma patients at risk for checkpoint-blockade-induced colitis. *Nat Commun* (2016) 7:10391. doi: 10.1038/ncomms10391
66. Lavasani S, Dzhambazov B, Nouri M, Fak F, Buske S, Molin G, et al. A novel probiotic mixture exerts a therapeutic effect on experimental autoimmune encephalomyelitis mediated by IL-10 producing regulatory T cells. *PloS One* (2010) 5(2):e9009. doi: 10.1371/journal.pone.0009009
67. Kwon HK, Lee CG, So JS, Chae CS, Hwang JS, Sahoo A, et al. Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. *Proc Natl Acad Sci USA* (2010) 107(5):2159–64. doi: 10.1073/pnas.0904055107
68. Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* (2018) 359(6371):97–103. doi: 10.1126/science.aan4236
69. Lopez P, Gonzalez-Rodriguez I, Sanchez B, Gueimonde M, Margolles A, Suarez A. Treg-inducing membrane vesicles from Bifidobacterium bifidum LMG13195 as potential adjuvants in immunotherapy. *Vaccine* (2012) 30 (5):825–9. doi: 10.1016/j.vaccine.2011.11.115
70. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* (2008) 453(7195):620–5. doi: 10.1038/nature07008
71. Erny D, Hrabe de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci* (2015) 18(7):965–77. doi: 10.1038/nn.4030
72. Messina JP, Gilkeson GS, Pisetsky DS. STIMULATION OF INVITRO MURINE LYMPHOCYTE-PROLIFERATION BY BACTERIAL-DNA. *J Immunol* (1991) 147(6):1759–64.
73. Yi AK, Klinman DM, Martin TL, Matson S, Krieg AM. Rapid immune activation by CpG motifs in bacterial DNA - Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J Immunol* (1996) 157(12):5394–402.
74. Ballas ZK, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J Immunol* (1996) 157(5):1840–5.
75. Cremonesi E, Governa V, Garzon JFG, Mele V, Amicarella F, Muraro MG, et al. Gut microbiota modulate T cell trafficking into human colorectal cancer. *Gut* (2018) 67(11):1984–94. doi: 10.1136/gutjnl-2016-313498
76. Valladares R, Sankar D, Li N, Williams E, Lai KK, Abdelgeliel AS, et al. Lactobacillus johnsonii N6.2 mitigates the development of type 1 diabetes in BB-DP rats. *PloS One* (2010) 5(5):e10507. doi: 10.1371/journal.pone.0010507
77. Chiba Y, Shida K, Nagata S, Wada M, Bian L, Wang C, et al. Well-controlled proinflammatory cytokine responses of Peyer's patch cells to probiotic Lactobacillus casei. *Immunology* (2010) 130(3):352–62. doi: 10.1111/j.1365-2567.2009.03204.x
78. Gostner JM, Geisler S, Stonig M, Mair L, Sperner-Unterweger B, Fuchs D. Tryptophan Metabolism and Related Pathways in Psychoneuroimmunology: The Impact of Nutrition and Lifestyle. *Neuropsychobiology* (2020) 79(1):89–99. doi: 10.1159/000496293
79. Schwarcz R, Bruno JP, Muchowski PJ, Wu H-Q. Kynurenines in the mammalian brain: when physiology meets pathology. *Nat Rev Neurosci* (2012) 13(7):465–77. doi: 10.1038/nrn3257

80. Fallarino I, Grohmann U, Vacca C, Bianchi R, Orabona C, Spreca A, et al. T cell apoptosis by tryptophan catabolism. *Cell Death Differ* (2002) 9 (10):1069–77. doi: 10.1038/sj.cdd.4401073
81. Raghavan S, Quiding-Jarbrink M. Regulatory T cells in gastrointestinal tumors. *Expert Rev Gastroenterol Hepatol* (2011) 5(4):489–501. doi: 10.1586/egh.11.44
82. Faith JJ, Ahern PP, Ridaura VK, Cheng J, Gordon JII. Identifying gut microbe-host phenotype relationships using combinatorial communities in gnotobiotic mice. *Sci Transl Med* (2014) 6(220):220ra11. doi: 10.1126/scitranslmed.3008051
83. Feuerer M, Hill JA, Mathis D, Benoist C. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol* (2009) 10 (7):689–95. doi: 10.1038/ni.1760
84. Kotler DP, Francisco A, Clayton F, Scholes JV, Orenstein JM. SMALL INTESTINAL INJURY AND PARASITIC DISEASES IN AIDS. *Ann Internal Med* (1990) 113(6):444–9. doi: 10.7326/0003-4819-113-6-444
85. Bengmark S, Jeppsson B. GASTROINTESTINAL SURFACE PROTECTION AND MUCOSA RECONDITIONING. *J Parenteral Enteral Nutr* (1995) 19 (5):410–5. doi: 10.1177/0148607195019005410
86. Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, et al. CD4 (+) Regulatory T Cells Control T(H)17 Responses in a Stat3-Dependent Manner. *Science* (2009) 326(5955):986–91. doi: 10.1126/science.1172702
87. Elyaman W, Bradshaw EM, Uyttenhove C, Dardalhon V, Awasthi A, Imitola J, et al. IL-9 induces differentiation of T(H)17 cells and enhances function of FoxP3(+) natural regulatory T cells. *Proc Natl Acad Sci USA* (2009) 106(31):12885–90. doi: 10.1073/pnas.0812530106
88. Roager HM, Licht TR. Microbial tryptophan catabolites in health and disease. *Nat Commun* (2018) 9:3249. doi: 10.1038/s41467-018-05470-4
89. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, et al. Tryptophan Catabolites from Microbiota Engage Aryl Hydrocarbon Receptor and Balance Mucosal Reactivity via Interleukin-22. *Immunity* (2013) 39(2):372–85. doi: 10.1016/j.immuni.2013.08.003
90. Hendrikx T, Schnabl B. Indoles: metabolites produced by intestinal bacteria capable of controlling liver disease manifestation. *J Internal Med* (2019) 286 (1):32–40. doi: 10.1111/joim.12892
91. Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med* (2008) 14(3):275–81. doi: 10.1038/nm1710
92. Basu R, O'Quinn DB, Silberger DJ, Schoeb TR, Fouser L, Ouyang W, et al. Th22 Cells Are an Important Source of IL-22 for Host Protection against Enteropathogenic Bacteria. *Immunity* (2012) 37(6):1061–75. doi: 10.1016/j.immuni.2012.08.024
93. Russell SL, Gold MJ, Hartmann M, Willing BP, Thorson L, Wlodarska M, et al. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep* (2012) 13(5):440–7. doi: 10.1038/embor.2012.32
94. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. *Science* (2011) 331(6015):337–41. doi: 10.1126/science.1198469
95. Karimi K, Inman MD, Bienenstock J, Forsythe P. Lactobacillus reuteri-induced Regulatory T cells Protect against an Allergic Airway Response in Mice. *Am J Respir Crit Care Med* (2009) 179(3):186–93. doi: 10.1164/rccm.200806-951OC
96. Pardoll D. Cancer and the Immune System: Basic Concepts and Targets for Intervention. *Semin Oncol* (2015) 42(4):523–38. doi: 10.1053/j.seminoncol.2015.05.003
97. Beck KE, Blansfield JA, Tran KQ, Feldman AL, Hughes MS, Royal RE, et al. Enterocolitis in patients with cancer after antibody blockade of cytotoxic T-lymphocyte-associated antigen 4. *J Clin Oncol* (2006) 24(15):2283–9. doi: 10.1200/JCO.2005.04.5716
98. Zitvogel L, Ma Y, Raoult D, Kroemer G, Gajewski TF. The microbiome in cancer immunotherapy: Diagnostic tools and therapeutic strategies. *Science* (2018) 359(6382):1366–70. doi: 10.1126/science.aar6918
99. Pitt JM, Vétizou M, Gomperts Boneca I, Lepage P, Chamaillard M, Zitvogel L. Enhancing the clinical coverage and anticancer efficacy of immune checkpoint blockade through manipulation of the gut microbiota. *OncoImmunology* (2016) 6(1):e1132137. doi: 10.1080/2162402X.2015.1132137
100. Perfetto SP, Chattopadhyay PK, Roederer M. Innovation - Seventeen-colour flow cytometry: unravelling the immune system. *Nat Rev Immunol* (2004) 4 (8):648–U5. doi: 10.1038/nri1416
101. Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. *Nat Rev Immunol* (2002) 2(4):251–62. doi: 10.1038/nri778
102. Lin H, Wei S, Hurt EM, Green MD, Zhao L, Vatan L, et al. Host expression of PD-L1 determines efficacy of PD-L1 pathway blockade-mediated tumor regression (vol 128, pg 805, 2018). *J Clin Invest* (2018) 128(4):1708–8. doi: 10.1172/JCI120803
103. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* (2009) 139(3):485–98. doi: 10.1016/j.cell.2009.09.033
104. Xie R, Jiang P, Lin L, Jiang J, Yu B, Rao J, et al. Oral treatment with Lactobacillus reuteri attenuates depressive-like behaviors and serotonin metabolism alterations induced by chronic social defeat stress. *J Psychiatr Res* (2020) 122:70–8. doi: 10.1016/j.jpsychires.2019.12.013
105. Forsythe P, Inman MD, Bienenstock J. Oral treatment with live Lactobacillus reuteri inhibits the allergic airway response in mice. *Am J Respir Crit Care Med* (2007) 175(6):561–9. doi: 10.1164/rccm.200606-821OC
106. Munn DH, Sharma MD, Lee JR, Jhaver KG, Johnson TS, Keskin DB, et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. *Science* (2002) 297(5588):1867–70. doi: 10.1126/science.1073514
107. Liu K, Yang Y, Chen Y, Li S, Gong Y, Liang Y. The therapeutic effect of dendritic cells expressing indoleamine 2,3-dioxygenase (IDO) on an IgA nephropathy mouse model. *Int Urol Nephrol* (2020) 52(2):399–407. doi: 10.1007/s11255-019-02365-1
108. Cavaillon JM. CYTOKINES AND MACROPHAGES. *Biomed Pharmacother* (1994) 48(10):445–53. doi: 10.1016/0753-3322(94)90005-1
109. Bleuze M, Auger J-P, Lavagna A, Gisch N, Gottschalk M, Segura M. In vitro characterization of granulocyte-colony stimulating factor (G-CSF) production by dendritic cells and macrophages during Streptococcus suis infection. *Immunobiology* (2020) 225(4):151979. doi: 10.1016/j.imbio.2020.151979
110. Denning TL, Wang YC, Patel SR, Williams IR, Pulendran B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nat Immunol* (2007) 8 (10):1086–94. doi: 10.1038/ni1511
111. Wenzel TJ, Gates EJ, Ranger AL, Klegeris A. Short-chain fatty acids (SCFAs) alone or in combination regulate select immune functions of microglia-like cells. *Mol Cell Neurosci* (2020) 105:103493. doi: 10.1016/j.mcn.2020.103493
112. Hengge UR, Benninghoff B, Ruzicka T, Goos M. Topical immunomodulators–progress towards treating inflammation, infection, and cancer. *Lancet Infect Dis* (2001) 1(3):189–98. doi: 10.1016/S1473-3099(01)00095-0
113. Shimada S, Yano O, Tokunaga T. INVIVO AUGMENTATION OF NATURAL-KILLER CELL-ACTIVITY WITH A DEOXYRIBONUCLEIC-ACID FRACTION OF BCG. *Japanese J Cancer Res* (1986) 77(8):808–16.
114. Yamamoto S, Yamamoto T, Shimada S, Kuramoto E, Yano O, Kataoka T, et al. DNA FROM BACTERIA, BUT NOT FROM VERTEBRATES, INDUCES INTERFERONS, ACTIVATES NATURAL-KILLER-CELLS AND INHIBITS TUMOR-GROWTH. *Microbiol Immunol* (1992) 36 (9):983–97. doi: 10.1111/j.1348-0421.1992.tb02102.x
115. Zhou W, Sonnenberg GF. Activation and Suppression of Group 3 Innate Lymphoid Cells in the Gut. *Trends Immunol* (2020) 41(8):721–33. doi: 10.1016/j.it.2020.06.009
116. Qi H, Li Y, Yun H, Zhang T, Huang Y, Zhou J, et al. Lactobacillus maintains healthy gut mucosa by producing L-Ornithine. *Commun Biol* (2019) 2:171. doi: 10.1038/s42003-019-0424-4
117. Belkacem N, Serafini N, Wheeler R, Derrien M, Boucinha L, Couesnon A, et al. Lactobacillus paracasei feeding improves immune control of influenza infection in mice. *PloS One* (2017) 12(9):e184976. doi: 10.1371/journal.pone.0184976
118. Zheng DW, Chen Y, Li ZH, Xu L, Li CX, Li B, et al. Optically-controlled bacterial metabolite for cancer therapy. *Nat Commun* (2018) 9(1):1680. doi: 10.1038/s41467-018-03233-9

119. Wu RY, Pasyk M, Wang B, Forsythe P, Bienenstock J, Mao YK, et al. Spatiotemporal maps reveal regional differences in the effects on gut motility for Lactobacillus reuteri and rhamnosus strains. *Neurogastroenterol Motil* (2013) 25(3):e205–14. doi: 10.1111/nmo.12072
120. West CL, Stanisz AM, Mao YK, Champagne-Jorgensen K, Bienenstock J, Kunze WA. Microvesicles from Lactobacillus reuteri (DSM-17938) completely reproduce modulation of gut motility by bacteria in mice. *PloS One* (2020) 15(1):e0225481. doi: 10.1371/journal.pone.0225481
121. Cook MT, Tzortzis G, Charalampopoulos D, Khutoryanskiy VV. Microencapsulation of probiotics for gastrointestinal delivery. *J Control Release* (2012) 162(1):56–67. doi: 10.1016/j.jconrel.2012.06.003
122. Dong Q-Y, Chen M-Y, Xin Y, Qin X-Y, Cheng Z, Shi L-E, et al. Alginate-based and protein-based materials for probiotics encapsulation: a review. *Int J Food Sci Technol* (2013) 48(7):1339–51. doi: 10.1111/ijfs.12078
123. Gombotz WR, Wee SF. Protein release from alginate matrices. *Adv Drug Delivery Rev* (2012) 64:194–205. doi: 10.1016/j.addr.2012.09.007
124. de Barros JMS, Scherer T, Charalampopoulos D, Khutoryanskiy VV, Edwards AD. A laminated polymer film formulation for enteric delivery of live vaccine and probiotic bacteria. *J Pharm Sci* (2014) 103(7):2022–32. doi: 10.1002/jps.23997
125. Yeung TW, Ucok EF, Tiani KA, McClements DJ, Sela DA. Microencapsulation in Alginate and Chitosan Microgels to Enhance Viability of Bifidobacterium longum for Oral Delivery. *Front Microbiol* (2016) 7:494. doi: 10.3389/fmicb.2016.00494
126. Dafe A, Etemadi H, Dilmaghani A, Mandavinia GR. Investigation of pectin/starch hydrogel as a carrier for oral delivery of probiotic bacteria. *Int J Biol Macromolecules* (2017) 97:536–43. doi: 10.1016/j.ijbiomac.2017.01.060
127. Mei L, He F, Zhou R-Q, Wu C-D, Liang R, Xie R, et al. Novel Intestinal-Targeted Ca-Alginate-Based Carrier for pH-Responsive Protection and Release of Lactic Acid Bacteria. *ACS Appl Mater Interf* (2014) 6(8):5962–70. doi: 10.1021/am501011j
128. de Barros JM, Lechner T, Charalampopoulos D, Khutoryanskiy VV, Edwards AD. Enteric coated spheres produced by extrusion/spheronization provide effective gastric protection and efficient release of live therapeutic bacteria. *Int J Pharm* (2015) 493(1-2):483–94. doi: 10.1016/j.ijpharm.2015.06.051
129. Wuerth R, Hoermannsperger G, Wilke J, Foerst P, Haller D, Kulozik U. Protective effect of milk protein based microencapsulation on bacterial survival in simulated gastric juice versus the murine gastrointestinal system. *J Funct Foods* (2015) 15:116–25. doi: 10.1016/j.jff.2015.02.046
130. Damodharan K, Palaniyandi SA, Yang SH, Suh JW. Co-encapsulation of lactic acid bacteria and prebiotic with alginate-fenugreek gum-locust bean gum matrix: Viability of encapsulated bacteria under simulated gastrointestinal condition and during storage time. *Biotechnol Bioprocess Eng* (2017) 22(3):265–71. doi: 10.1007/s12257-017-0096-1
131. Wang J, Nickerson MT, Low NH, Van Kessel AG, Plaizier J. Efficacy of pea protein isolate–alginate encapsulation on viability of a probiotic bacterium in the porcine digestive tract. *Can J Anim Sci* (2016) 97(2):214–22. doi: 10.1139/cjas-2016-0090
132. Shi L-E, Li Z-H, Li D-T, Xu M, Chen H-Y, Zhang Z-L, et al. Encapsulation of probiotic Lactobacillus bulgaricus in alginate-milk microspheres and evaluation of the survival in simulated gastrointestinal conditions. *J Food Eng* (2013) 117(1):99–104. doi: 10.1016/j.jfoodeng.2013.02.012
133. de Barros JM, Costabile A, Charalampopoulos D, Khutoryanskiy VV, Edwards AD. Evaluating and optimizing oral formulations of live bacterial vaccines using a gastro-small intestine model. *Eur J Pharm Biopharm* (2016) 102:115–22. doi: 10.1016/j.ejpb.2016.03.010
134. Li W, Zhu Y, Ye F, Li B, Luo X, Liu S. Probiotics in cellulose houses: Enhanced viability and targeted delivery of Lactobacillus plantarum. *Food Hydrocolloids* (2017) 62:66–72. doi: 10.1016/j.foodhyd.2016.07.019
135. Huq T, Fraschini C, Khan A, Riedl B, Bouchard J, Lacroix M. Alginate based nanocomposite for microencapsulation of probiotic: Effect of cellulose nanocrystal (CNC) and lecithin. *Carbohydr Polymers* (2017) 168:61–9. doi: 10.1016/j.carbpol.2017.03.032
136. Diab R, Canilho N, Pavel IA, Haffner FB, Girardon M, Pasc A. Silica-based systems for oral delivery of drugs, macromolecules and cells. *Adv Colloid Interface Sci* (2017) 249:346–62. doi: 10.1016/j.cis.2017.04.005
137. Zheng H, Gao M, Ren Y, Lou R, Xie H, Yu W, et al. An improved pH-responsive carrier based on EDTA-Ca-alginate for oral delivery of Lactobacillus rhamnosus ATCC 53103. *Carbohydr Polym* (2017) 155:329–35. doi: 10.1016/j.carbpol.2016.08.096
138. Chowdhuri S, Cole CM, Devaraj NK. Encapsulation of Living Cells within Giant Phospholipid Liposomes Formed by the Inverse-Emulsion Technique. *Chembiochem* (2016) 17(10):886–9. doi: 10.1002/cbic.201500643
139. Lin J, Yu W, Liu X, Xie H, Wang W, Ma X. In Vitro and in Vivo characterization of alginate-chitosan-alginate artificial microcapsules for therapeutic oral delivery of live bacterial cells. *J Biosci Bioeng* (2008) 105 (6):660–5. doi: 10.1263/jbb.105.660
140. Li W, Luo X, Song R, Zhu Y, Li B, Liu S. Porous Cellulose Microgel Particle: A Fascinating Host for the Encapsulation, Protection, and Delivery of Lactobacillus plantarum. *J Agric Food Chem* (2016) 64(17):3430–6. doi: 10.1021/acs.jafc.6b00481
141. Li H, Van Thi Thuy H, Turner MS, Dhital S. Encapsulation of Lactobacillus plantarum in porous maize starch. *Lwt Food Sci Technol* (2016) 74:542–9. doi: 10.1016/j.lwt.2016.08.019
142. Nualkaekul S, Lenton D, Cook MT, Khutoryanskiy VV, Charalampopoulos D. Chitosan coated alginate beads for the survival of microencapsulated Lactobacillus plantarum in pomegranate juice. *Carbohydr Polymers* (2012) 90(3):1281–7. doi: 10.1016/j.carbpol.2012.06.073
143. Anselmo AC, McHugh KJ, Webster J, Langer R, Jaklenec A. Layer-by-Layer Encapsulation of Probiotics for Delivery to the Microbiome. *Adv Mater* (2016) 28(43):9486–90. doi: 10.1002/adma.201603270
144. Wei OY, Chen HM, Jones ML, Metz T, Haque T, Martoni C, et al. Artificial cell microcapsule for oral delivery of live bacterial cells for therapy: design, preparation, and in-vitro characterization. *J Pharm Pharm Sci* (2004) 7 (3):315–24.
145. Varankovich NV, Khan NH, Nickerson MT, Kalmokoff M, Korber DR. Evaluation of pea protein-polysaccharide matrices for encapsulation of acid-sensitive bacteria—-104. *Food Res Int* (2015) 70:118–24. doi: 10.1016/j.foodres.2015.01.028
146. Cook MT, Tzortzis G, Khutoryanskiy VV, Charalampopoulos D. Layer-by-layer coating of alginate matrices with chitosan–alginate for the improved survival and targeted delivery of probiotic bacteria after oral administration. *J Mater Chem B* (2013) 1(1):52–60. doi: 10.1039/C2TB00126H
147. D'Orazio G, Di Gennaro P, Boccarusso M, Presti I, Bizzaro G, Giardina S, et al. Microencapsulation of new probiotic formulations for gastrointestinal delivery: in vitro study to assess viability and biological properties. *Appl Microbiol Biotechnol* (2015) 99(22):9779–89. doi: 10.1007/s00253-015-6853-1
148. Turroni F, Duranti S, Milani C, Lugli GA, van Sinderen D, Ventura M. Bifidobacterium bifidum: A Key Member of the Early Human Gut Microbiota. *Microorganisms* (2019) 7(11):544. doi: 10.3390/microorganisms7110544
149. Kato K, Odamaki T, Mitsuyama E, Sugahara H, Xiao JZ, Osawa R. Age-Related Changes in the Composition of Gut Bifidobacterium Species. *Curr Microbiol* (2017) 74(8):987–95. doi: 10.1007/s00284-017-1272-4
150. Odamaki T, Bottacini F, Kato K, Mitsuyama E, Yoshida K, Horigome A, et al. Genomic diversity and distribution of Bifidobacterium longum subsp. longum across the human lifespan. *Sci Rep* (2018) 8(1):85. doi: 10.1038/s41598-017-18391-x
151. Huda MN, Ahmad SM, Alam MJ, Khanam A, Kalanetra KM, Taft DH, et al. Bifidobacterium Abundance in Early Infancy and Vaccine Response at 2 Years of Age. *Pediatrics* (2019) 143(2):e20181489. doi: 10.1542/peds.2018-1489
152. Oh NS, Joung JY, Lee JY, Kim Y. Probiotic and anti-inflammatory potential of Lactobacillus rhamnosus 4B15 and Lactobacillus gasseri 4M13 isolated from infant feces. *PloS One* (2018) 13(2):e0192021. doi: 10.1371/journal.pone.0192021
153. Capurso L. Thirty Years of Lactobacillus rhamnosus GG: A Review. *J Clin Gastroenterol* (2019) 53 Suppl 1:S1–S41. doi: 10.1097/MCG.0000000000001170
154. Haffner FB, Girardon M, Fontanay S, Canilho N, Duval RE, Mierzwa M, et al. Core–shell alginate@silica microparticles encapsulating probiotics—74. *J Mater Chem B* (2016) 4(48):7929–35. doi: 10.1039/C6TB02802K
155. Tenaillon O, Skurnik D, Picard B, Denamur E. The population genetics of commensal Escherichia coli. *Nat Rev Microbiol* (2010) 8(3):207–17. doi: 10.1038/nrmicro2298
156. Ahmadzadeh M, Farshdari F, Nematollahi L, Behdani M, Mohit E. Anti-HER2 scFv Expression in Escherichia coli SHuffle((R))T7 Express Cells:

Effects on Solubility and Biological Activity. *Mol Biotechnol* (2020) 62(1):18–30. doi: 10.1007/s12033-019-00221-2
157. Cebeci A, Gürakan C. Properties of potential probiotic Lactobacillus plantarum strains. *Food Microbiol* (2003) 20(5):511–8. doi: 10.1016/S0740-0020(02)00174-0
158. Wang J, Ji H, Wang S, Liu H, Zhang W, Zhang D, et al. Probiotic Lactobacillus plantarum Promotes Intestinal Barrier Function by Strengthening the Epithelium and Modulating Gut Microbiota. *Front Microbiol* (2018) 9:1953. doi: 10.3389/fmicb.2018.01953
159. Seddik HA, Bendali F, Gancel F, Fliss I, Spano G, Drider D. Lactobacillus plantarum and Its Probiotic and Food Potentialities. *Probiotics Antimicrob Proteins* (2017) 9(2):111–22. doi: 10.1007/s12602-017-9264-z
160. Chen H, Ouyang W, Jones M, Haque T, Lawuyi B, Prakash S. In-vitro analysis of APA microcapsules for oral delivery of live bacterial cells. *J Microencapsul* (2005) 22(5):539–47. doi: 10.1080/02652040500162162
161. Zhang H, Yang C, Zhou W, Luan Q, Li W, Deng Q, et al. A pH-Responsive Gel Macrosphere Based on Sodium Alginate and Cellulose Nanofiber for Potential Intestinal Delivery of Probiotics. *ACS Sustain Chem Eng* (2018) 6 (11):13924–31. doi: 10.1021/acssuschemeng.8b02237
162. Aryana KJ, Olson DW. A 100-Year Review: Yogurt and other cultured dairy products. *J Dairy Sci* (2017) 100(12):9987–10013. doi: 10.3168/jds.2017-12981
163. Ryan PM, Ross RP, Fitzgerald GF, Caplice NM, Stanton C. Sugar-coated: exopolysaccharide producing lactic acid bacteria for food and human health applications. *Food Funct* (2015) 6(3):679–93. doi: 10.1039/C4FO00529E
164. Hill D, Sugrue I, Tobin C, Hill C, Stanton C, Ross RP. The Lactobacillus casei Group: History and Health Related Applications. *Front Microbiol* (2018) 9:2107. doi: 10.3389/fmicb.2018.02107
165. Jacouton E, Chain F, Sokol H, Langella P, Bermudez-Humaran LG. Probiotic Strain Lactobacillus casei BL23 Prevents Colitis-Associated Colorectal Cancer. *Front Immunol* (2017) 8:1553. doi: 10.3389/fimmu.2017.01553
166. Zhao C, Zhu Y, Kong B, Huang Y, Yan D, Tan H, et al. Dual-Core Prebiotic Microcapsule Encapsulating Probiotics for Metabolic Syndrome. *ACS Appl Mater Interfaces* (2020) 12(38):42586–94. doi: 10.1021/acsami.0c13518
167. Dilnawaz F, Singh A, Mohanty C, Sahoo SK. Dual drug loaded superparamagnetic iron oxide nanoparticles for targeted cancer therapy. *Biomaterials* (2010) 31(13):3694–706. doi: 10.1016/j.biomaterials.2010.01.057
168. Li X, He Y, Hou J, Yang G, Zhou S. A Time-Programmed Release of Dual Drugs from an Implantable Trilayer Structured Fiber Device for Synergistic Treatment of Breast Cancer. *Small* (2020) 16(9):1902262. doi: 10.1002/smll.201902262
169. Lammers KM, Brigidi P, Vitali B, Gionchetti P, Rizzello F, Caramelli E, et al. Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripheral blood mononuclear cells. *FEMS Immunol Med Microbiol* (2003) 38(2):165–72. doi: 10.1016/S0928-8244(03)00144-5
170. Lai W-F, Lin MC-M. Nucleic acid delivery with chitosan and its derivatives. *J Controlled Release* (2009) 134(3):158–68. doi: 10.1016/j.jconrel.2008.11.021
171. Zhao QQ, Chen JL, Han M, Liang WQ, Tabata Y, Gao JQ. Combination of poly(ethylenimine) and chitosan induces high gene transfection efficiency and low cytotoxicity. *J Biosci Bioeng* (2008) 105(1):65–8. doi: 10.1263/jbb.105.65
172. Kiang T, Bright C, Cheung CY, Stayton PS, Hoffman AS, Leong KW. Formulation of chitosan-DNA nanoparticles with poly(propyl acrylic acid) enhances gene expression. *J Biomater Science Polymer Edition* (2012) 15 (11):1405–21. doi: 10.1163/1568562042368112
173. Zhang Y, Chen J, Zhang Y, Pan Y, Zhao J, Ren L, et al. A novel PEGylation of chitosan nanoparticles for gene delivery. *Biotechnol Appl Biochem* (2007) 46 (Pt 4):197–204. doi: 10.1042/BA20060163
174. Weecharangsan W, Opanasopit P, Ngawhirunpat T, Apirakaramwong A, Rojanarata T, Ruktanonchai U, et al. Evaluation of chitosan salts as non-viral gene vectors in CHO-K1 cells. *Int J Pharm* (2008) 348(1-2):161–8. doi: 10.1016/j.ijpharm.2007.07.011
175. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. *EMBO Rep* (2006) 7(7):688–93. doi: 10.1038/sj.embor.7400731
176. Qian J, Wang C, Wang B, Yang J, Wang Y, Luo F, et al. The IFN-gamma/PD-L1 axis between T cells and tumor microenvironment: hints for glioma anti-PD-1/PD-L1 therapy. *J Neuroinflamm* (2018) 15(1):290. doi: 10.1186/s12974-018-1330-2
177. Mimura K, Teh JL, Okayama H, Shiraishi K, Kua LF, Koh V, et al. PD-L1 expression is mainly regulated by interferon gamma associated with JAK-STAT pathway in gastric cancer. *Cancer Sci* (2018) 109(1):43–53. doi: 10.1111/cas.13424
178. Garcia-Diaz A, Shin DS, Moreno BH, Saco J, Escuin-Ordinas H, Rodriguez GA, et al. Interferon Receptor Signaling Pathways Regulating PD-L1 and PD-L2 Expression. *Cell Rep* (2017) 19(6):1189–201. doi: 10.1016/j.celrep.2017.04.031
179. Mo X, Zhang H, Preston S, Martin K, Zhou B, Vadalia N, et al. Interferon-gamma Signaling in Melanocytes and Melanoma Cells Regulates Expression of CTLA-4. *Cancer Res* (2018) 78(2):436–50. doi: 10.1158/0008-5472.CAN-17-1615
180. Gao J, Shi LZ, Zhao H, Chen J, Xiong L, He Q, et al. Loss of IFN-gamma Pathway Genes in Tumor Cells as a Mechanism of Resistance to Anti-CTLA-4 Therapy. *Cell* (2016) 167(2):397–404.e9. doi: 10.1016/j.cell.2016.08.069

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REVIEW
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The "Gum–Gut" Axis in Inflammatory Bowel Diseases: A Hypothesis-Driven Review of Associations and Advances

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In modern medicine, the oral cavity has often been viewed as a passive conduit to the upper airways and gastrointestinal tract; however, its connection to the rest of the body has been increasingly explored over the last 40 years. For several diseases, the periodontium and gingiva are at the center of this oral-systemic link. Over 50 systemic conditions have been specifically associated with gingival and periodontal inflammation, including inflammatory bowel diseases (IBD), which have recently been elevated from simple "associations" to elegant, mechanistic investigations. IBD and periodontitis have been reported to impact each other's progression *via* a bidirectional relationship whereby chronic oral or intestinal inflammation can impact the other; however, the precise mechanisms for how this occurs remain unclear. Classically, the etiology of gingival inflammation (gingivitis) is oral microbial dysbiosis in the subgingival crevice that can lead to destructive periodontal disease (periodontitis); however, the current understanding of gingival involvement in IBD is that it may represent a separate disease entity from classical gingivitis, arising from mechanisms related to systemic inflammatory activation of niche-resident immune cells. Synthesizing available evidence, we hypothesize that once established, IBD can be driven by microbiomial and inflammatory changes originating specifically from the gingival niche through saliva, thereby worsening IBD outcomes and thus perpetuating a vicious cycle. In this review, we introduce the concept of the "gum–gut axis" as a framework for examining this reciprocal relationship between the periodontium and the gastrointestinal tract. To support and explore this gum–gut axis, we 1) provide a narrative review of historical studies reporting gingival and periodontal manifestations in IBD, 2) describe the current understanding and advances for the gum–gut axis, and 3) underscore the importance of collaborative treatment and research plans between oral and GI practitioners to benefit this patient population.

Keywords: gum–gut, oral–gut, microbiome, gingivitis, periodontitis, Crohn's disease, ulcerative colitis, inflammatory bowel disease

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INTRODUCTION

A Bidirectional Influence of Oral and Systemic Health

The oral cavity serves as the entry point to the gastrointestinal tract and is also continuous with the nasal cavity and the skin of the face (1). While it certainly functions as a conduit for the movement of food, fluids, and air, this space has been revealed to be a diverse collection of tissues that are harmoniously integrated into the vital functions of communication, defense, feeding, breathing, and early digestion (2–4). Diseases of these tissues range from innocuous, seriously disabling, or even lethal. Indeed, they were recognized as such by Hippocrates, who cataloged oral diseases as part of—not separate from—the whole body (5, 6). Despite this ancient perspective and recent efforts by healthcare leaders to breakdown longstanding barriers, the concept of oral medicine existing separate from general medicine persists (7–9).

While many pathologies are confined to the oral cavity itself, there has been increasing exploration of the links between oral diseases and systemic health. We and others hypothesize that this is a bidirectional link, centered around the generalized influence of chronic inflammation. Specifically, there exist several oral-systemic axes in which inflammatory diseases of the oral cavity can lead to dysbiosis, which then influences the systemic disease course—and vice versa. For example, numerous systemic diseases demonstrate manifestations in the oral cavity (**Figure 1A**). In particular, several nutritional deficiencies and systemic diseases involving the skin, hematopoietic system, immune system, endocrine system, connective tissues, lungs, liver, kidneys, and the gastrointestinal tract are known to demonstrate a diverse array of bony, glandular, connective tissue, and mucosal manifestations in the oral cavity (10, 11). While the purpose of this review is not to catalog all known or suspected oral-systemic axes, it is evident that these links are likely underappreciated in both oral health care and in medicine.

The mechanistic underpinnings for each of these oral-systemic axes are limited. The reasons for this are numerous, but one important consideration is that they can present unpredictably and are known to present in specific oral niches. The soft tissues of the oral cavity are heterogeneous, comprised of transitions between masticatory and lining mucosal tissues, taste and tactile papillae on the dorsal tongue, major and minor salivary glands, palatine and lingual tonsillar tissues, and the tooth and its supporting periodontium—comprised of the periodontal ligament, cementum, alveolar bone, and the gingiva (12). When considering the involvement of systemic disease in the oral cavity, the uniqueness of these oral tissues inadvertently predilects some sites to be more or less likely to display the influence of systemic effects. One of the best examples of this is in inflammatory bowel diseases (IBD), which can present in nearly every niche, including the lymph nodes, buccal mucosa (cheek lining), tongue, lips, teeth, and periodontium (13). The most common oral manifestations of IBD involve the buccal mucosa and the gingiva, both of which can display severe, chronic inflammatory lesions. This is not surprising, as the anatomy of the periodontium makes this a susceptible site for frequent dysbiosis and chronic inflammation.

Among all oral niches, the periodontium has been the most often explored as key to the oral-systemic link (14, 15), and the influence of chronic inflammatory diseases of the periodontium (gingivitis and periodontitis) on systemic healthy was formalized as the term "periodontal medicine" to describe these gum-systemic links in the 1990s (16–20). To date, over 50 diseases have been associated with

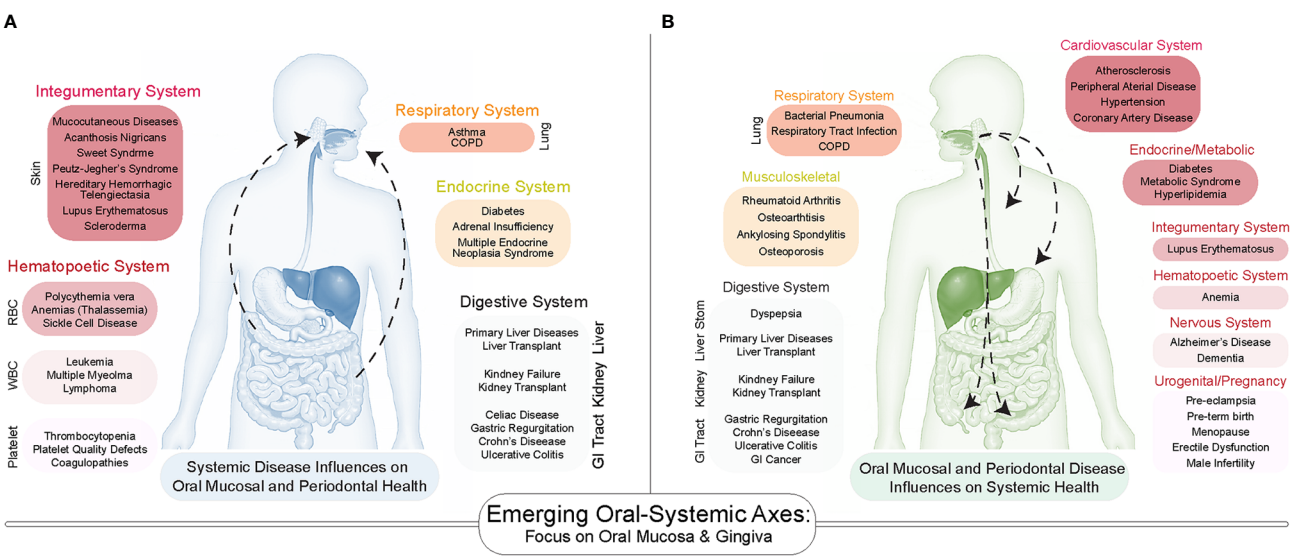

FIGURE 1 | A Bidirectional Influence of oral and systemic health. **(A)** Emerging associations between systemic disease on oral health include diseases of the skin, lungs, gastrointestinal tract as well as endocrine and hematopoietic systems. **(B)** The number of systemic diseases impacted by oral inflammatory diseases of the periodontium (gingivitis and periodontitis) continues to increase as more studies are conducted. Many of the same systems that influence oral health are influenced by periodontal health.

gingivitis/periodontitis (21), including atherosclerotic disease (22), adverse pregnancy outcomes (23), type I and type II diabetes (24, 25), metabolic syndrome (26), and inflammatory bowel diseases (**Figure 1B**) (27). The directionality and causality of these associations remain to be elucidated, but recent mechanistic investigations into the oral-systemic link in IBD have recently provide clues into the interworking of this axis (28, 29). This review will focus on the periodontium as a specialized tissue niche that not only displays involvement in IBD but may also be able to seed those local changes to the distant gut to exacerbate the course of IBD. Throughout this review, we will synthesize what is known across many fields to provide support for an emerging oral–gut link in IBD. However, we will emphasize the idea of this phenomenon likely being uniquely associated with gingival and periodontal inflammation and thus, we introduce the concept of the "gum–gut axis" for the first time as a framework for examining the reciprocal relationship between the periodontium and the gastrointestinal tract (i.e., gut-to-gum influences and gum-to-gut influences). To support and explore this emerging gum–gut axis, we 1) provide a narrative review of historical studies reporting gingival and periodontal manifestations in IBD, 2) describe the current understanding and advances for the gum–gut axis, and 3) underscore the importance of collaborative treatment and research plans between oral and GI scientists to benefit this patient population.

BACKGROUND

Periodontitis and Periodontal Disease as a Manifestation of IBD

Currently, about 40% of all US adults older than 30 have some form of periodontal disease (30), and ~11% of the world's population is currently diagnosed with a severe form of the disease (31). Periodontal diseases are immune-mediated, chronic disorders of the periodontium. Based on the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions, diseases of these tooth-supporting tissues are now classified into gingivitis, as well as three major periodontal disease categories: (a) periodontitis, (b) necrotizing periodontal diseases, and (c) periodontitis as a manifestation of systemic disease (32). Periodontal diseases typically originate in the gingiva as inflammation before causing progressive alveolar bone destruction (33, 34). Over many years of work, it is known that gingivitis and periodontitis are caused by a shift from a healthy to a dysbiotic biofilm in the subgingival crevice or "pocket" (35, 36). Once established, periodontal diseases display extensive disease heterogeneity but are commonly defined by chronic and destructive periodontal inflammation that can lead to loss of tooth-supporting tissues and a lower quality of life (37). These diseases are often diagnosed after 30 years old (38); however, it is also important to note that severe gingivitis can occur at any age, even in children.

Similarly, IBD represents a group of immune-mediated, chronic inflammatory disorders of the gastrointestinal tract. They are typically characterized into two primary disease types: Crohn's disease (CD) and ulcerative colitis (UC). Among these diseases, there is signifcant heterogeneity within these subtypes, and many more IBD phenotypes likely exist than have been defined. The incidence of IBD in the US and Europe has recently stabilized (39, 40), yet the US still has about 25% of the world's cases when age-standardized metrics are utilized (40). Pediatric IBD comprises about 25% of all cases, and these patients typically have more aggressive disease, with up to 34% requiring surgery within 10 years of diagnosis (41, 42). Modern twin studies suggest the heritability of both CD and periodontitis is around 0.3, though slightly less for UC (43, 44). Both IBD and periodontitis are actively being investigated for perturbations of host genetics, the microbiome, environment, diet, and inflammatory/immune cell subtypes as potentially explaining disease progression (45, 46).

Gingival and Periodontal Manifestations of IBD

While the extraintestinal manifestations of IBD may involve the skin, eyes, and joints (47, 48), oral involvement can occur in up to 50% of all cases. For pediatric IBD, oral manifestations— often in the gingiva—may be present as high as 80%, with higher prevalence in males and CD (49–52). Though reports vary, it has been suggested that up to 25% of IBD cases present with oral symptoms before any intestinal involvement (48, 53); that said, the most frequently reported oral manifestations are the appearance of "cobblestoning" and ulceration of the oral mucosa (13) as well as chronic, severe inflammation of the gingiva/periodontium (54). It is noteworthy that while IBD patients commonly display severe gingival inflammation and hypertrophy, lesions of the gingiva can also be subtle. For example, mild inflammation of the gingival margin may present as a subclinical lesion (marginal gingivitis) (55); moreover, there is significant heterogeneity of gingival lesions generally, despite controlling for similar patient demographics (56). Even among trained providers, diagnosing gingival disease can be challenging and time-consuming (49, 57, 58); thus, it is likely that the prevalence of gingival manifestations of IBD at a "person-level" is likely underreported. To explore the gum–gut axis in IBD, it is imperative that we first explore the pathogenesis of gingivitis and compare it to what is known about gingivitis in IBD.

Classical Gingivitis *vs* IBD-Induced Gingivitis

In health, gingivae appear pink in color, firm to palpation, and occasionally stippled with no obvious pathology (i.e., bone resorption; **Figure 2**) (59). Diseases of the gingiva are often associated with inflammation of the gums/gingiva, and by definition, involve only the soft tissues of the periodontal attachment (60). Like periodontitis, it has been shown that gingivitis is caused by local dysbiosis of the subgingival crevice (i.e., in the subgingival microbiome) (61, 62); however, the gingival inflammation observed in IBD patients does not appear to follow this well-known pathogenesis in all cases. Extracting from recent reports, it appears that gingival and periodontal inflammation in IBD may not be biofilm-induced, but rather, biofilm-exacerbated (27). This would establish a

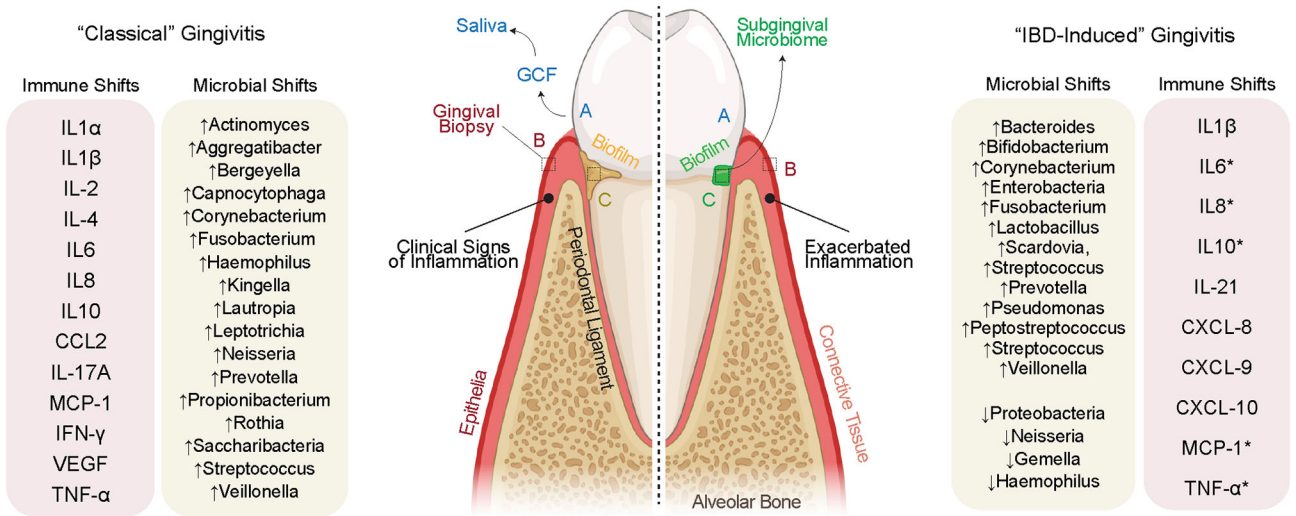

FIGURE 2 | Evidence for IBD-induced gingivitis as a separate disease entity. (Left) "Classically" defined gingivitis involves well-defined increases to pro-inflammatory cytokines secondary to defined shifts in the microbiome. The inflammation clinically documented in IBD often discordant with biofilm deposits on the tooth surface, suggesting a role for the systemic inflammation of IBD to cause oral inflammation in parallel with or independent of biofilm deposits. This inflammation may itself shift the oral microbiome which then may cause further gingival inflammation. A sampling of the gingival crevicular fluid (GCF) and saliva (biofluids), gingival tissues (full-thickness biopsies), and subgingival microbes (microbiome) allows for a detailed understanding of the inflammatory and microbiomial shifts shared and unique to these possibly unique diseases.

paradigm for the **gum–gut** axis whereby the treatment modalities and understanding of the disease cascade may not necessarily follow these classic studies and raises the possibility that IBD-induced gingivitis could be a different disease entity altogether. This requires further exploration.

In classical gingivitis (or simply "gingivitis" for this review), inflammation of the gingival margin presents with erythema as well as increases in the inflammatory infiltrate (63). Understanding the differences between gingivitis and IBD-induced gingivitis pathogenesis will be important but also challenging. This is because gingivitis is simultaneously reversible and increasingly prevalent as we age; ~1/3 of all 3-year-olds, ~2/3 of 5-year-olds, and >90% of young adults have gingivitis (64). Gingival and periodontal diseases can present from to mild to severe, often historically classified as initial, early, established, and advanced lesions (62). The "initial" lesion of gingivitis is not detectable clinically but has been shown to occur within 2–4 days after biofilm accumulation. This results in an increase in gingival crevicular fluid flow, vasoactive compound release, neutrophil migration into the gingival crevice, and finally the release of effector cytokines to induce more inflammation (59). Through progressive changes, the classical "early" lesion is established within a week of biofilm accumulation and is defined by an increased relative abundance of lymphocytes and macrophages (65). While the response of the host tissues is increasingly heterogeneous when examining individuals (66), the more mature established gingival lesion is defined by a significant increase in B and plasma cells (59).

In gingivitis, the subgingival microbiome undergoes a measurable shift in taxa that elicit an immune response [see list in **Figure 2**; (67)]. While these species are found in the subgingival microbiome, the host inflammatory response in gingivitis is now thought to be a result of alterations to microbiomial abundance, richness, and interspecies interaction (67). While there is important person-to-person variation, the result of sustained gingivitis in susceptible individuals is periodontitis, often defined by an increased presence of *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Aggregatibacter actinomycetemcomitans* (68). While these concepts of microbiomial "ecology" are still being explored in gingivitis, the similarities and differences for IBD-induced gingivitis are only now emerging after years of case reports and association studies.

THE HISTORY OF THE "GUM–GUT" AXIS IN IBD

Classical Case Reports

Like periodontal diseases, the signs and symptoms of IBD have been alluded to throughout human history. UC was first described in case series from the late 19th century, and separately, CD in 1932 (69, 70). Periodontal disease classifications have been dynamically revised over the years and multiple classifications have been proposed, starting as early as the late 19th century; however, it was not until 1942 that a classification paradigm was based on the principles of pathology detailed "gingivitis" or "periodontitis" (7, 71). As early classifications of these diseases became better understood and

more widely disseminated, only then were studies of gingival and periodontal manifestations in IBD even possible to conduct (**Figure 3**).

Oral manifestations of IBD were first reported in the 1950s and initially focused on aphthous ulceration (72), though many reviews reference a description of oral granulomatous inflammation from Dudeney and Todd in 1969 as the first report of oral involvement in IBD (73). Over the following decades, detailed case reports were published that highlighted the diversity of oral manifestations in IBD, generally highlighting the most severe lesions (74–77). For example, one early case description detailed an account of a pediatric CD patient who developed unilateral granulomatous inflammation in the buccal mucosa 6 years after the initial diagnosis (73). Over the next few years, similar case reports were published expanding these observations of oral manifestations in IBD patients to soon detail other sites such as the lips and the hard palate (75, 78, 79).

Among these studies, gingival and periodontal manifestations of IBD were also reported in the literature, often including patients presenting with severe gingivitis (73, 80, 81). In these studies, similarities were noted between the pathogenesis of periodontitis and IBD. The connection between IBD and specific manifestations in the gingiva was documented around the same time as other manifestations. For example, a case report from 1972 detailed findings from a pediatric CD patient who presented with gingival hyperplasia on the entire maxillary anterior teeth (80). The tissues were described as 5–6 mm "pseudopockets", suggestive of a severe hyperplastic phenotype that worsened periodically. While there was no bony involvement—and therefore not truly periodontitis—this was a unique manifestation of IBD and suggested that chronic, severe gingivitis in IBD patients may itself undergo temporal exacerbation and remission. As early as 1978, the first studies suggesting severe periodontitis might be associated with IBD, which were published (82–84). These reports provided some of the earliest evidence for a "gum–gut" axis in IBD and highlighted gaps in knowledge related to the long-term impact of this chronic periodontal inflammation over the life span.

Association Studies

Building on these early case reports, cross-sectional studies were subsequently designed to search for positive associations among IBD and periodontal afflicted individuals. Some of the earliest studies on these gum–gut associations focused on the gingiva of children. A later study specifically assessed gingival inflammation in children and adolescents (aged 4–18 years old) (85). Interestingly, this report focused on a remission cohort of patients receiving treatment with immune-modulating medications (i.e., anti-TNF; others). Even though the matched healthy controls and IBD subjects displayed similar oral health habits, IBD patients self-reported a significantly higher incidence of bleeding gums when brushing and had higher gingival inflammation scores upon clinical examination. Based on the Community Periodontal Index of Treatment Needs (CPITN) index, none of the IBD patients were determined to have healthy periodontal tissues (0/55 subjects), and about 2/3 had a high need for periodontal treatment (scores >1). This highlights that the use of clinical indices of periodontal disease severity can better define the strength of the gum–gut axis.

Studies like these presented substantial support for a gum–gut axis in which gingival inflammation was a primary manifestation of IBD, even in children. Ultimately, longitudinal studies will be required to truly understand the impact of early gum inflammation on the gut in the long-term. Because such prospective cohort studies are yet to be conducted, most association studies have focused on adult cross-sectional populations. However, few of these studies have documented critical variables such as the length of time since IBD diagnosis or

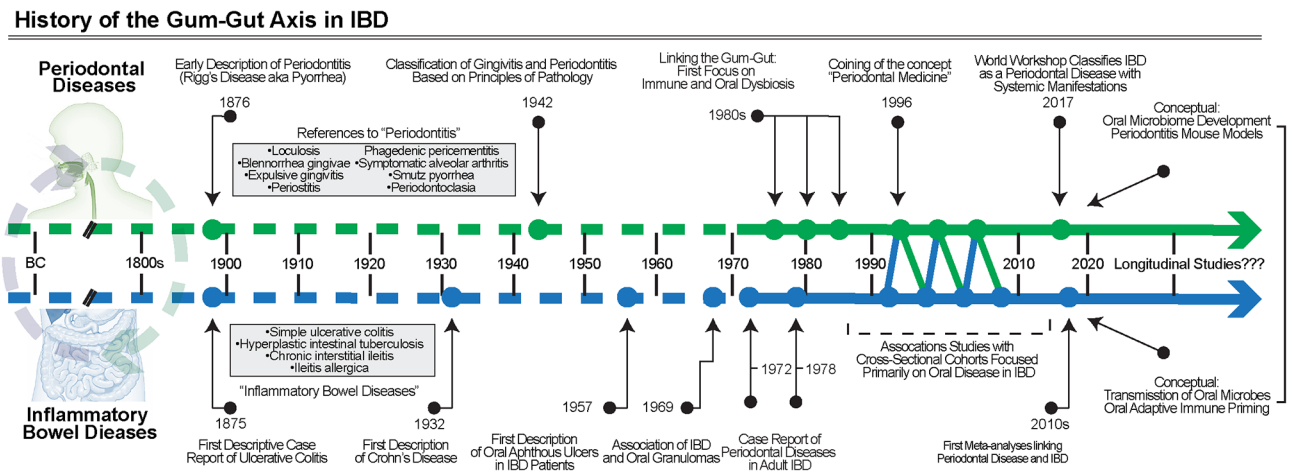

FIGURE 3 | The History of the gum–gut axis in IBD. Over many centuries, the chronic inflammatory diseases of the gut and periodontium were noted but not classified into defined disease entities until the late 1800s and early 1900s. First with case reports, then association studies, and finally clinical trials using biosampling primarily of the oral cavity, the evidence for the bidirectional relationship became better understood. More studies are required, including longitudinal cohorts, to understand the temporal associations and to further test causality.

measures such as the severity over time, which does lead to some difficulty in interpreting results. For example, a 1991 study showed that IBD patients display a higher prevalence—but a decreased severity—of periodontitis (86). A 2006 report assessed periodontitis in patients diagnosed with IBD using a case-control study design and again showed a trend toward lower severity, but higher periodontal disease prevalence, in IBD patients (87). These unexpected findings support the idea that that gingivitis and IBD-induced gingivitis are unique and underscore that while IBD-induced gingival inflammation is often documented to be more clinically severe, this mechanism of inflammation may be less likely to lead to the tissue destruction seen in periodontitis (88, 89). This is at odds with what is known for the classical dysbiotic-driven disease course and suggests more is left to learn for both IBD and healthy individuals.

Other studies have also found consistent positive associations between periodontitis and IBD compared to healthy controls, using standard indices of periodontal inflammation in adults (88, 89). For example, a 2013 article detailed 113 patients with IBD compared to healthy controls and found all clinical markers of periodontitis such as bleeding on probing, loss of clinical attachment, and pocket depth were each increased in both UC and CD patients (90). Additional case-control studies have demonstrated that IBD is positively associated with common clinical indices of gingivitis and periodontitis and that moderate-to-severe CD activity correlates with clinical indices of periodontal disease (90). A recent meta-analysis has aggregated these cohorts and summarized the results of six studies (total: 599 IBD patients and 448 control subjects), revealing significant positive risk associations for periodontitis in CD (3.64; 95% CI: 2.33–5.67) and UC (5.37; 95% CI: 3.30–8.74) (91). Another meta-analysis of 9 cross-sectional studies similarly found risk ratios of 4.55 for having periodontitis in IBD patients (92). These documented associations of periodontitis and IBD may be established long before periodontitis manifests, and when considering the mechanisms that drive the gum–gut axis, there is an unmet need to understand how IBD-induced periodontal manifestations are compared to classically understood concepts in IBD, including tissue barrier, inflammation, and microbiomial dysbiosis.

FACTORS THAT DRIVE THE GUM–GUT AXIS

Gingival Barrier

The gingiva, which is comprised of oral epithelium, connective tissue, blood and lymphatic vessels, smooth muscle, and fibroblasts, forms around each tooth as it erupts. The overlying oral epithelia fuse with the specialized, tooth-associated reduced enamel epithelium, the latter which establishes the soft tissue attachments to the non-shedding surfaces of the tooth *via* hemidesmosomes. This developmental process transforms the stratified squamous epithelia into both a sulcular epithelium and tooth-associated junctional epithelia over 4 years to form the gingival sulcus—and physical barrier—circumferentially around each primary and permanent tooth (59, 93, 94). It is at this critical niche where a dynamic host structural, host immune, and microbial relationship influences disease initiation and progression. Despite its innate and acquired immune defense, it remains incredibly susceptible to disease. These host tissues, however, are also increasingly considered an immune organ, performing functions necessary to withstand assaults from the mechanical forces associated with mastication and from frequent microbiomial shifts (95, 96). Even in health, more inflammatory cells are detected at the gingival barrier compared to other oral sites, suggesting this site may be able to readily attract immune cells to mount a defense against a shifting subgingival microbiome (97, 98).

This barrier site displays an underappreciated and poorly understood epithelial and mesenchymal cell heterogeneity (99, 100) but has an incredible ability to tolerate the stress of the oral environment, to regenerate after periodontal surgeries (94), and has an emerging role in immune cell recruitment and "crosstalk" (101, 102). This makes this niche one of the most dynamic in the oral cavity. These vulnerable cells are held together by cell-cell adhesions such as cadherins (CH1, CDH3), desmogleins (DSG1, DSG2, DSG3), desmocollins (DSC1, DSC2, DSC3), and tight junctions (TJP1, OCLN, CLDN1, CLDN7, CLDN10, CLDN12) (103, 104). The expression of these adhesion genes is heterogeneous by gingival epithelial cell type, which is of interest considering recent work that suggests the fine-tuning of immunity occurs from the structural cells present in various body niches (38). Also of relevance is the impact of diet on the host barriers in the oral cavity and intestine, which suggest vitamins C primarily from fruits may play a potential protective effect across the gastrointestinal tract (105–108). There is much still to be learned about the impact of the environment, diet, and niche-specific immune interactions in periodontal diseases. A broader and deeper understanding of these modifiers will help us understand how gingival inflammation in IBD is related to gingivitis. However, studies to date have only focused on host inflammation and oral microbiome in IBD.

Systemic Inflammation

Some of the earliest studies that considered the mechanisms of the gum–gut axis include reports in the late 1970s/1980s from Lamster et al. and Engel et al. which suggested functional changes to the immune system may play a common and important role in patients with CD and periodontitis pathogenesis (83, 84, 109). These studies provide a framework on how this nascent field would approach studies of the gum–gut axis in IBD: through profiling the inflammatory milieu, sampling the oral microbiome, or both; however, this association is not causality, even considering more sensitive methods of sampling and profiling.

For convenience, saliva has often been collected as a surrogate for localized tissue inflammation, though profiling of the gingival crevicular fluid around the inflamed gingiva would be assumed to provide a clearer signal. Despite this, salivary sampling in CD patients found elevated effector cytokines (IL-1β, IL-6, IL-8, and MCP-1; IL-1β and TNF-α) compared to healthy controls (110).

IL-6, IL-1β, and TNF-α are elevated in salivary sampling comparing active/exacerbated to inactive/remission CD patients, whereas active UC patients demonstrated increased IL-4, IL-10, and IL-21 (111, 112). Buccal mucosal sampling of pediatric CD patients revealed higher chemokines (CXCL-8, -9, -10) compared to healthy children and even adults with CD, suggesting possibly unique signatures of oral manifestations of pediatric IBD even when compared to adults (113). For one of the few studies that sampled the site of proposed inflammation, a study of gingival biopsies in adults with IBD and periodontitis revealed no differences between UC and CD but did show that across all sites, gingiva express IL-17A, IL-17F, IL-22, IL-25, IL-33, IL-10, and IFN-γ compared to the intestinal biopsies. These findings suggest that there may be unique inflammatory profiles in the oral niches compared to the intestine even at baseline, which establishes a challenging framework for studies interested in defining the local inflammatory profile of distant sites in the same subject (114).

Microbial Dysbiosis

The first study to explore oral microbiomial shifts in IBD patients was by Van Dyke et al. who tied periodontal disease to oral dysbiosis. In this classic study, the oral microbiota was characterized in the periodontal pockets of two groups: 1) those with IBD and periodontitis and 2) those with IBD and only IBD-associated oral manifestations of soft tissues (115). The investigators found that the microbiota of IBD-associated periodontal pockets was unique compared to patients with IBD *but no oral involvement*., enriched with a unique microbiota of mostly small, gram-negative rods that were thought to be *Wolinella* (now: *Campylobacter*—a genus that is also associated with periodontal and other gastrointestinal diseases) (116, 117). Engel et al. focused on a case of severe, generalized periodontitis in a recent diagnosis of CD and also found a host of well-known periodontal pathogens in their patient, including *Porphyromonas gingivalis, Tannerella forsythia, and Campylobacter rectus* (84).

Since gingival inflammation in IBD does not appear consistent with plaque accumulation, an interesting question is whether the systemic inflammation caused by IBD leads to changes of the subgingival microbiome, thus leading to dysbiosis that exacerbates oral inflammation. There is precedent for chronic inflammatory diseases like diabetes, systemic lupus erythematosus, or rheumatoid arthritis to influence the oral microbiome (118). Moreover, while mechanistically unclear, disturbances of salivary and oral mucosal microbiomes have been reported in two unique mouse models of colitis (DSS and *Citrobacter rodentium* infection) and this has been validated in humans (27, 119). The ability of IBD to induce inflammation due to the recognition of shared epitopes across the body has been suggested as a possible mechanism for extraintestinal manifestations of IBD (120, 121). Arguably, extraintestinal manifestations may be induced by local gut dysbiosis that causes a broad adaptive immune response that leading to the recognition of these epitopes in sites like the gingiva (121).

Building off of what had been thought of as dysbiosis in adult IBD and periodontitis patients, a 2012 study assessed the oral microbiome of the tongue and buccal mucosa in 114 healthy, pediatric IBD subjects using 16S rRNA profiling (122). This study found less overall oral microbiome diversity in CD compared to healthy controls—but not in UC. Tongue microbiomes revealed increased *Spirochetes*, *Bacteroides*, and *Synergistes* as well as decreased Firmicutes and Fusobacterium in IBD subjects compared to healthy controls. Another pediatric study sampled the subgingival microbial niche in 46 healthy and 35 CD patients (ages between 6 and 17 years old) before and after 8 weeks of pharmacotherapy (123). A majority of these cases displayed resolution of intestinal inflammation, but in treatment naïve CD patients, this study found increased *Capnocytophaga*, *Rothia*, and *Saccharibacteria* in the gingiva of IBD patients compared to healthy controls. When CD patients who had received antibiotic therapy were compared to a CD treatment-naïve cohort, the treatment cohort was shown to have decreased periopathogenic genera such as *Fusobacterium* and *Porphyromonas*, suggesting that some IBD treatments may have on reducing inflammation through changes to the oral microbiome.

Other IBD studies have found increased *Bacteroides*, *Prevotella*, and *Veillonella* and decreased *Proteobacteria, Neisseria, Gemella*, and *Haemophilus* when comparing the salivary microbiomes of IBD patients to healthy controls (110). A sampling of the subgingival microbiome in young and old patients have also shown unique oral dysbiotic signatures in IBD patients with gingivitis (increased *Prevotella*, *Peptostreptococcus*, *Streptococcus* species) or periodontitis (increased *Bacteroides*, *Campylobacter, and Porphyromonas species)* (124). A recent study of the salivary microbiome of UC and CD found increased diversity and enrichment of *Streptococcus* and *Enterobacteria* in UC and *Veillonella* in CD when either was compared to healthy controls (125). This group was able to identify distinct "oral ecotypes" for UC and CD; each was not defined by clinical characteristics or disease severity (125). The "indicator species" of these ecotypes varied over time but included *Corynebacterium* and *Acinetobacter* for UC and *Lactobacillus*, *Bifidobacterium*, *Scardovia*, *Streptococcus*, and *Pseudomonas* for CD. Ecotype 1 (CD) showed a specific enrichment of *Neisseria* and *Fusobacterium*. Furthermore, a recent study of the buccal mucosa in irritable bowel syndrome also revealed a decrease in Bacteroides and *Bacillus*, suggesting that gastrointestinal diseases other than IBD may influence the oral microbiome (126). Based on these studies, it appears that between pediatric and adult subjects, commonly increased taxa in untreated CD appear to include *Bacteroides, Campylobacter, Fusobacterium, Porphyromonas, Prevotella, and Veillonella*, which may provide important targets to better understand this gum–gut axis in future studies; however, the question of whether IBD-induced periodontal manifestations follow the same dysbiotic-immune paradigm as gingivitis and periodontitis remains unresolved.

DEFINING THE GUM–GUT AXIS

A Framework for the Understanding the Gum–Gut Axis

The decreasing cost and increasing sensitivity of high throughput assays to ask questions about host immunity across the life span

make this an especially ripe time to understand the gum–gut axis in IBD. There is still a great deal to learn, but we believe that elucidating this axis may have important long-term ramifications for improving the health of oral and gastrointestinal tissues and decreasing disease incidence by guiding and training our host immune systems for a more balanced immune response later in life.

These immune-microbiomial concepts are not new. For example, while preterm birth has been associated with dysregulated neonatal immunity, immune system "priming" in health is thought to occur through the birth canal from exposure to *Escherichia* and *Enterococcus* genera, as well as obligate/facultative anaerobes, including members of the Firmicutes and Bacteroidetes phyla and the *Bifidobacterium* genus. This narrow window for establishing long-term health is important for other mucosal tissues to help educate our mammalian immune systems (127). While immune priming has been recently shown to be important for the intestine through breastmilk (i.e., non-genetic inheritance of IgA), oral immune priming throughout neonatal oral microbiomial exposures remains to be fully explored (128). This will be even more important if early disturbances to oral immune priming are found to negatively affect gut immune priming (i.e., neonatal gum-to-gut influence; **Figure 4**).

We hypothesize that the uniqueness of the gingiva—from baseline immune profiles to the dynamic shifts of the microbiome—provides the possibility of connection to the gastrointestinal tract through the seeding of these products *via* gingival crevicular fluid and then *via* saliva. While a nascent field, the gum–gut axis also draws on a broad body of evidence. In the following sections, we will outline the interconnectedness of oral and gut microbiomial development and theorize how disturbances to these systems may influence disease. We will also emphasize saliva as a transmission vehicle for oral microbes and inflammatory cells/meditators to the lower gastrointestinal (**Figure 4**).

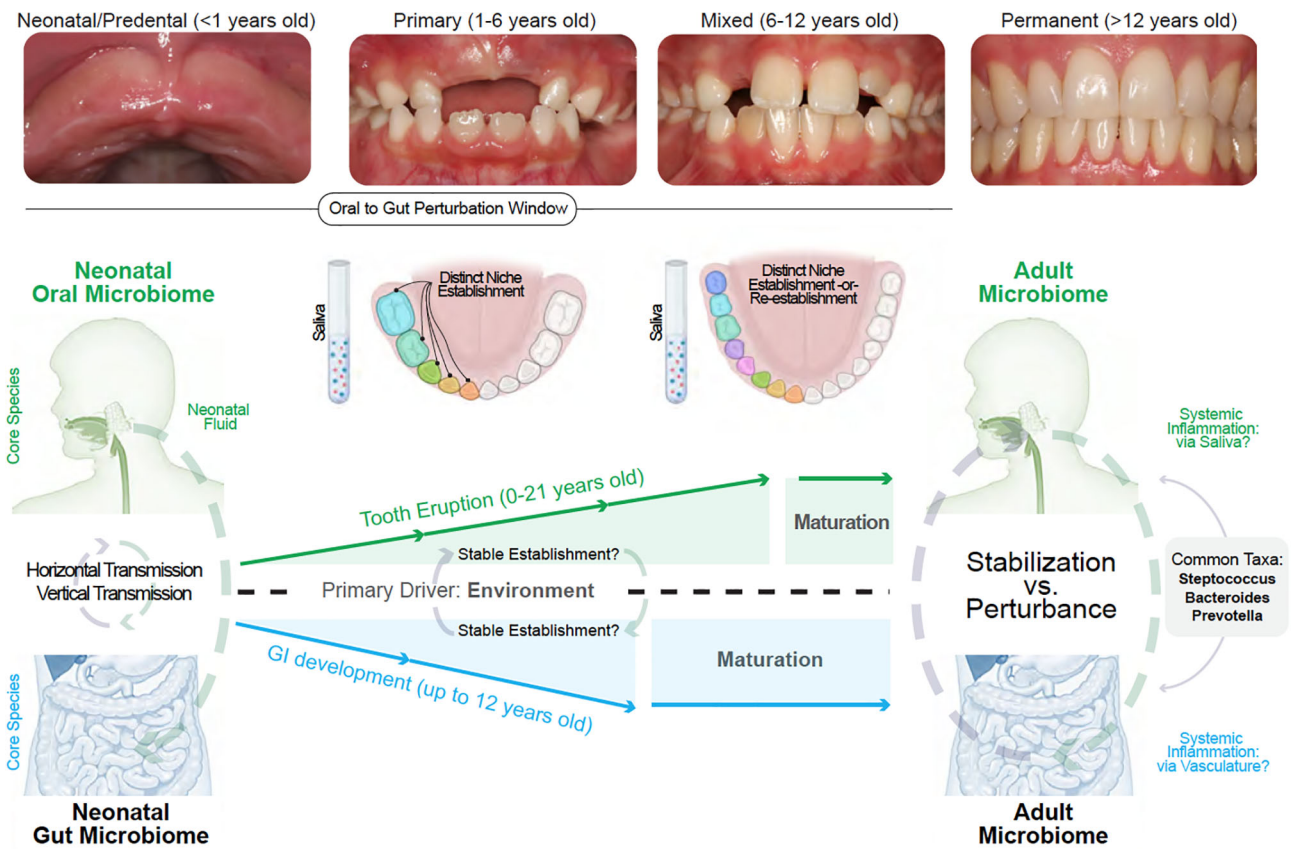

FIGURE 4 | Linking the gum–gut axis from birth to adulthood. Due to the uniqueness of each site, neonatal and adult oral and gut microbiomes do not resemble one another. However, a common feature is that both sites and their distinct niches are established with a nascent microbiome that diversifies during development. The oral microbiome stabilize at a later development timeframe when compared to the gut microbiome due to the shedding of primary teeth until about 12 years old and the subsequent eruption of permanent teeth. This provides a narrower window of gut microbiome vulnerability compared to the oral cavity. Both oral and gut microbiome diversification and stabilization are reportedly driven by environmental influences. The influence of the oral cavity to the lower gastrointestinal tract can occur *via* saliva and also *via* vasculature (local inflammation seeding to distant sites) whereas the primary mode of gut to oral influence is *via* the mechanisms of local-to-systemic inflammation such as the delivery of effector cytokines or activation of oral tissue-resident immune cells.

The Interconnectedness of Oral and Gut Microbiomial Establishment

The human microbiome consists of a dynamic relationship between the microbiota—namely, viruses, protozoa, fungi, archaea, and bacteria—and their human niches; these niches can be found on the skin, the reproductive organs, as well as the interconnected gastrointestinal and upper aerodigestive tracts (including the lungs, intestines, and oral cavity) (129, 130). We are just now beginning to understand how this "forgotten organ" is established and matures along with the development of its host (131). The gut microbiome has been well-studied; however, historically, the oral cavity was one of the first sites used in the discovery of human microbes by Van Leeuwenhoek over three centuries ago (132).

Currently, it is unknown exactly how many microbial species exist in the pediatric or adult human oral cavity, but the Human Oral Microbiome Database (http://homd.org/) lists over >150 genera, 700 species, and 1,300 strains, including an incredibly diverse mycobiome of almost 100 fungi genera, and nearly 5,000 biosynthetic gene clusters which produce small molecules to allow for microbial communication (133–136). The vast majority of oral microbiota are commensals—but also include symbiotes and pathogens—and as more strains are discovered, the more it is recognized how microbial diversity and, importantly, their interdependence is dynamic (137). Unsurprisingly, comparing adult oral and gut microbiomes reveals that they are more dissimilar than similar; however, there are some common taxa between the two, including *Streptococcus*, *Bacteroides*, and *Prevotella*. The relative abundance of *Streptococcus* and *Bacteroides* differ significantly, though *Prevotella* was found to be closely matched when considering a grouping of buccal mucosa and hard palate versus a stool sample (~3% abundance in both groups) (138, 139). Considering another grouping of the tongue, salivary, and oropharyngeal samples resulted in 4× fold relative detection of *Prevotella* compared to other oral and GI microbiomes; consistently, however, there is substantial heterogeneity of oral microbiomes when comparing niches core species.

Relevant to the gum–gut axis is how the oral and gut microbiomes develop and how these unique microbiomes evolve as we age (140). It is now thought that oral and gut colonization between living partners and between those partners' children is the direct influence of both horizontal and vertical transmission mechanisms, respectively (141–144). Oral microbiomes cluster more clearly when age and niche are considered; this is an important point to consider moving forward when thinking about oral–gut association studies (145). While the stable establishment of the host microbiomes has been shown to influence the health of the individual later in their life (146–148), whether this establishment occurs *in utero* remains controversial (149, 150). Currently, it is thought that, while *in utero* colonization may be possible, it is also restricted. Recent 16S studies of meconium have detected only 18 total taxa assumed to be from the gut, dominated by *Micrococcus* and *Lactobacillus* (151). How this relates to future oral and gut mucosal immunity remains to be discovered.

Though ecologically distinct and with their characteristic species, the oral and gut microbiomes both become more diverse after birth; however, the gut microbiome stabilizes earlier than the oral microbiome. This is likely due to the longer period of growth, development, and tooth eruption in the oral cavity in the first two decades of life (152, 153). This trend also follows for the intestinal immune system compared to the oral cavity (154). When considering the gum–gut axis, we do not currently know if and when "perturbation windows" to either microbiome may more effectively compromise the health of the oral and the intestinal microenvironment (**Figure 4**).

The establishment of stable oral microbiomes may also be critical for overall health in children; for example, *Bifidobacteria* are known to be an important component of a healthy gut microbiome—especially during lactation and breastfeeding—and have been shown to influence immune responses in the gut (155). Interestingly, recent work has shown that *Bifidobacteria* may predominantly seed the gut *via* neonatal oral fluid (156, 157). Another important question is how breastfeeding, which is filtered through the mouth, may simultaneously influence the development of both oral and gut microbiomes. For example, the neonatal gut virome has been shown to develop sequentially through breastfeeding (158), and it is known that the oral cavity can host several unique viromes that likely benefit from this mode of neonatal feeding (159).

Oral Microbiomial Development: Oral Mucosa, Subgingiva, and Saliva

To connect the gum to the gut, the goal of this section is to establish a logical link between predentate mucosal microbiomes, the subsequent emergence of teeth to establish the first subgingival microbiomes, and then to connect this site to the lower gastrointestinal tract through the oral biofluids around the tooth (via gingival crevicular fluid) and then the whole oral cavity (via saliva). We hypothesize that chronic disruption of the oral immune repertoire sets up a gum–gut axis whereby the gingival microbiota shift towards dysbiosis, establishing a positive feedback loop for a chronic lesion of effector immune cells that can travel and occasionally survive the journey in saliva to the rest of the body, including the lower gastrointestinal tract (**Figure 4**).

While the oral microbiome is often referred to as an individual entity, it is important to emphasize that there are several ways to think about the microbiomial niches within the oral cavity. For example, it is known that there is a 1) biogeographical (i.e., spatial) diversity of species within polymicrobial communities (160), 2) biofluidic diversity comparing salivary to the gingival crevicular fluid, 3) niche-by-niche diversity between oral mucosal sites (161), and even 4) anteroposterior diversity proposed to be caused by retrograde salivary flow during swallowing (162). Despite this vast heterogeneity, most single niches only contain about 5% of the total known species (137). Some species are common to multiple sites, each with a preference for various "landscape ecologies" of the oral cavity that support this niche heterogeneity over time (163). Recent work considering the site-specificity of adult oral

microbiomes has documented these unique species for the dorsal tongue mucosa, gingiva, and the enamel surfaces of teeth (161).

There is also the question of temporal heterogeneity. Our oral and gut microbiomes continually change throughout our life, but even the most primitive microbiomes in neonates are now thought to play an active role in our oral and gut development (164). While host genetics may contribute to microbiomial development in a niche, a recent twin study of oral microbiomial variation of the supragingival niche over 12 months suggests that environmental exposure may be the primary influence on microbes found in each niche (165). This has also been shown for salivary microbiomes in a cohort of children between 2 days and 5 years old (166). The development of microbiomes in the oral cavity is highly unique compared to the rest of the body; this is because, in children and adolescence, these microbiomes are often defined by the dentition "state". In neonates, this is often staged as a mouth with no teeth present (neonatal/predentate mucosa); toddlers display primary dentition; children display a mixed dentition, and teenagers display a permanent dentition with only adult teeth (**Figure 4**).

Temporally, predentate neonates display the most rudimentary oral mucosal microbiomes, but even at this timepoint, 50 genera have been identified, the majority of which appear to be facultative anaerobes and anaerobes (167). Primary colonizers (0–3 months old) include *Lactobacillus, Fusobacterium, Staphylococcus, Streptococcus*, and *Veillonella*; secondary colonizers include *Gemella, Granulicatella, Haemophilus*, and *Rothia.* Neonatal microbiomes also appear to vary at the species-level with only ~30 "core species" identified (167). At 3 months old, which is still before the first teeth erupt for most infants, predominant oral microbiomial phyla include *Saccharibacteria* (formerly *TM7)*, *Fusobacteria*, and *Actinobacteria*—but not in all children, again supporting the importance of environmental influence on microbiome development (168). Oral mucosal microbiomes have been shown to increase in diversity and richness along with the developmental progression of predentate to permanent (145). It has been reported that the neonatal oral mucosa displays a high microbiomial diversity but low richness (169). Predentate microbiomes are populated with *Bacteroides*, Firmicutes, *Eikenella, Eubacterium, Gemella*, *Granulicatella, Oribacterium, Proteobacteria, Selenomonas*, *Streptococcus*, and *Veillonella*.

It is on the foundation of this predentate mucosal microbiome that the subgingival microbiome is established, and there is a correlation between maternal and neonatal predentate core species. This is critical and sensitive. When mothers who were also smokers were studied (passive maternal smoking), an increase in periodontal pathogens such as *Campylobacter* and Fusobacterium were uniquely noted in the predentate microbiomes of their offspring. These are important to emphasize because they are also known IBD pathobionts, and a recent multicenter study found that passive maternal smoking had a dose-dependent association with the development of pediatric IBD (170). Additionally, maternal smoking habits in the perinatal period have been associated with developing IBD in their offspring (odds ratios of 5.32 for CD; 3.02 for UC) when matched to unexposed controls, and we hypothesize that these oral microbial alterations may play an active role in the exacerbation or acceleration of IBD.

Once the predentate microbiome is established, the subgingival microbiome forms as teeth erupt through that oral mucosa to form the subgingival crevice. This niche is unique in the mouth, protecting from oxygen and low redox potential for Gram-negative anaerobes (96, 171). Studies focused solely on the gingival pocket have detected nearly 500 different species in the subgingival biofilms attached to the tooth and with few species dominating in adults (172). This unique anatomy results in a complex microbiota even in children, with only about 1/3 of genera shared with the predentate microbiomes (167). In this niche, several taxa are common to primary, mixed, and permanent dentition states, including *Actinomyces*, *Capnocytophaga*, *Campylobacter*, *Corynebacterium*, Fusobacterium, *Gemella*, *Granulicatella, Haemophilus. Kingella, Porphyromonas, Prevotella, Streptococcus, Terrahaemophilus*, and *Veillonella* (167).

As permanent teeth erupt, the richness of species increases as does oral microbiomial "personalization". This is the site of microbiomial dysbiosis in gingivitis that can occur in children and adults. In both classical and IBD-associated gingivitis, the gingival crevicular fluid flows outward through the junctional epithelial attachment into the subgingival crevice and eventually the saliva. It is thought that this crevicular fluid may also serve as nutrition for the oral microbiome. As more species colonize the subgingival space, gingival crevicular flow rates and pH have been shown to increase (173, 174). A few recent studies on adults have assessed this fluid for its microbiome; however, because it flows into saliva and likely contains free-floating microbes from the developing subgingival biofilms, its signature appears more as an intermediate between these two niches (175–177).

Changes to the salivary microbiome during oral development have also been examined (167). Saliva is not solely a microbiome source but also contains factors that support commensal microbiomial development and maintenance (178). Like other oral microbiomes, the salivary microbiomial composition is also defined by ecological succession (168). The salivary microbiome has very few "core species" shared between these developmental stages (167); however, the concept of "early colonizers" has been explored and suggests that *Streptococcus* and *Veillonella* appear first, followed by *Neisseria.* What appears most consistent is that salivary microbiomes are closely aligned with the dentition state; thus, the salivary microbiome clusters with the predentate microbiome in neonates and clusters more with the primary teeth stage microbiomes in toddlers, etc. This results in salivary microbiomes that increase in diversity as primary teeth first erupt, are shed, and permanent teeth again erupt. For example, neonatal salivary taxa are dominated by *Streptococcus, Veillonella, and Gemella*—much like the neonatal microbiome. When the primary dentition erupts, similarities to the maternal oral microbiomes become less apparent, which accompanies the detection of *Actinomyces*, *Corynebacterium, Granulicatella*, Fusobacterium, *Haemophilus, Neisseria*, and *Rothia* (167). This reflects the ability of saliva to be sampled as a gestalt—but not as a specific— readout.

Linking the Oral Microenvironment to the Gut Through Saliva

Currently, there are several proposed mechanisms for how periodontal diseases may influence distant sites like the intestines. These links include dissemination of periopathogens and inflammatory mediators like TNF, IL-1β, and IL-6 systemically through the bloodstream (179) Though many studies have suggested a potential for chronic periodontal inflammation and local oral dysbiosis to influence other body sites, few studies have determined causality (i.e., whether this is occurring *via* the distant effects of inflammation from chronic gingivitis/periodontitis or transmission of oral pathogens to distant sites). The latter considers a role for translocation of the subgingival microbiome; however, one group recently examined the gut microbiomes of patients with health, gingivitis, and periodontitis. They found that chronic oral inflammation was associated with less alpha-diversity in the gut microbiome, and though some oral taxa could be detected from the stool of each patient cohort, no clear trends for oral taxa enrichment emerged in this pilot—thus pointing to the possible influence of gut microbiome composition through oral inflammation (180).

From the recent literature, the gum–gut axis appears to be inherently linked through saliva, which can deliver enzymes, effector cytokines, free-floating and keratinocyte-bound bacteria, and subpopulations of viable inflammatory cells such as neutrophils, lymphocytes, and macrophages to distant sites. Saliva also contains mucus (comprised of water, lipids, and proteins such as mucins) which can protect these contents from the acidic contents of the stomach for survival along the gastrointestinal tract (181). About ~1 to 1.5 L of saliva is produced daily per person, and this contains millions of bacteria that are traditionally not known to colonize distant intestinal sites in health (182). While there is an interest in whether and how dysbiotic subgingival microbiomes could lead to the subsequent release of pro-inflammatory cytokines, this is a burgeoning field of study. Recent studies in mice showed a role for known periopathogen *Porphyromonas gingivalis* in perpetuating systemic inflammation after oral administration in mice; this led to endotoxemia, altered the gut microbiome, decreased insulin resistance, and altered tight junction expression in the ileum (183). Other studies have linked *Atopobium parvulum, Campylobacter concisus, Fusobacterium nucleatum, Fusobacterium varium*, and *Staphylococcus aureus* to gastrointestinal disease, but whether these species are colonizing the intestine or indirectly eliciting chronic immune responses remains to be seen (184).

Several interesting studies have put forth the first evidence of transmission and colonization of oral microbes to the upper aerodigestive tract and also to the intestine (gum to gut influence). In health, there is evidence for oral microbiomial contribution to the oropharyngeal, esophagus, and gastric microbiomes (185). For example, an early study of the distal esophagus found 13 genera common to all samples, including *Streptococcus*, *Prevotella*, and *Veillonella*; most species-level OTUs were determined to be similar or identical to those of the oral cavity (186). This distant transmission is not specific to the GI tract as colonization and succession of the lung microbiome are associated with cystic fibrosis progression in infants and children (187). This work showed colonization of the lungs by oral microbes was possible even in 2-year-old children, with a significant abundance of *Streptococcus*, *Prevotella*, and *Veillonella*—identical to those found in the distal esophagus. Periopathogen taxa such as Fusobacterium and *Porphyromonas* were detected as well in some progressing groups, supporting the potential role for known periopathogens transmission in disease progression in a distant site.

This transmission phenomenon is also observed in the lower gastrointestinal tract (188, 189). Given these findings, what was once thought to be a rare event—the colonizing of distant microbiomial niches by oral microbes—appears to be more commonplace than once appreciated. For example, recent work assessing both salivary and stool samples primarily from healthy adults estimated that >10% of oral species may transmit *via* an oral-fecal route throughout the entire GI tract (190). This demonstrates a previously underappreciated niche-to-niche colonization pipeline. Other studies have reported many oral microbes found in the intestinal tissues of adult patients suffering from IBD, such as *Aggregatibacter, Campylobacter, Enterobacteria*, Fusobacterium*, Gemella, Neisseria, Pasteurella, Peptostreptococcus*, and *Streptococcus*. Many of these are associated with gingivitis (184). These findings are supported by well-designed studies in mice that have demonstrated competition for the GI niche by oral and traditional gut microbes (191), as well as other recent work that has demonstrated the establishment of oral microbes in the gastrointestinal tracts of patients afflicted with colorectal cancers and adult IBD (185, 188, 192, 193). A recent study of IBD found colon biopsies were abundantly colonized by periopathogens such as Fusobacterium*, Peptostreptococcus, Staphylococcus*, and *Streptococcus* (194).

Mechanistic studies using gnotobiotic mice have also shown a role for resident *Klebsiella* spp. in the saliva of IBD patients to colonize an already dysbiotic colon, leading to a significant inflammatory response through Type 1 T helper (TH1) cells in the local gut microenvironment (28). Whether *Klebsiella* spp. are truly a pathogenic link between the oral cavity and the gut or merely a demonstration of pathogenic colonization and immune-mediated exacerbation of IBD in mice remains to be elucidated. However, there is an exciting future ahead, especially when considering a recent study that utilized a ligature model in mice to induce periodontal inflammation. This led to subgingival dysbiosis with increased *Bacteroides*, *Enterobacteriaceae*, and *Staphylococcus*. *Enterobacteriaceae* were also found in the gut microbiome, again suggesting that oral microbes were able to colonize the intestine, and also exacerbate, established colitis. This study found that this was dived *via* oral niche primed Th17 cells with tropism for the gut (29). While these studies have not yet answered how these systems are explicitly linked, there is increasingly strong evidence for oral dysbiosis and localized gingival/periodontal inflammation eliciting and exacerbating and immune responses in the gut. While much of this review

has focused on the oral gingival niche as one half of the axis (gut to gut inflammation and subsequent gut to gum influence), this reflects the state of the field. The local gut niche in IBD with and without chronic oral inflammation is a nascent field with many more mechanistic and clinical studies needed.

DISCUSSION

There remain several challenges to formally establishing our proposed gum–gut axis. In particular, the heterogeneity of IBD and periodontal disease—as well as the temporal nature of each to exhibiting periods of activation and remission—make this association difficult to establish until better subtyping and disease activity for each disease can be more clearly ascribed (**Figure 5**). It remains critical that we continue to learn more about the pathogenesis of both oral and GI diseases so that we can also better understand how they influence each other. This will allow for the development of future tools to improve both our oral and gastrointestinal health throughout life. Additionally, while oral manifestations are not often the primary concern for treatment in many systemic diseases like IBD, their oral manifestations may provide a "window to the rest of the body", serving as an easily accessible site to aid in the diagnosis or to serve as a functional readout of disease activity (195). This could include frequent oral sampling through treatment naïve IBD patients to predict disease activity or to determine the efficacy of IBD biologics/biosimilars chairside before and during the critical first months after deciding on a particular IBD pharmacotherapeutic.

In parallel with a non-invasive sampling of the oral cavity in longitudinal studies, it is clear that model organisms such as mice are proving vital to provide supporting evidence for the gum–gut axis. This type of work is supported by a recent resource of oral microbiomial development that has cataloged predentate, eruption, and post-eruption stages in mice. This provides a framework for studies about niche establishment, dysbiosis, and the long-term consequences and resistance to gum–gut disease. Additionally, ileitis models (such as SAMP1/YitFc mice) and DSS models of colitis are reported to show oral mucosal inflammation and inflammatory bone loss that mimics periodontitis in mice and could be useful for these investigations (196, 197). While it is interesting to postulate about which oral taxa could take residence and cause, reactivate, or exacerbate IBD, further studies are required to understand how gut microbial growth rates, antibiotic resistance (i.e., resistomes), microbial gene expression, and metabolomics all come together to influence IBD development and perpetuate IBD (198–201).

While much work remains to be done, there is an exciting future for collaborative efforts between GI and oral health care providers to answer these questions. This will require biosampling both oral and intestinal sites to correlate dysbiosis and inflammatory changes across niches. It is encouraging to see the progress of the NIH Human Microbiome Project over the last

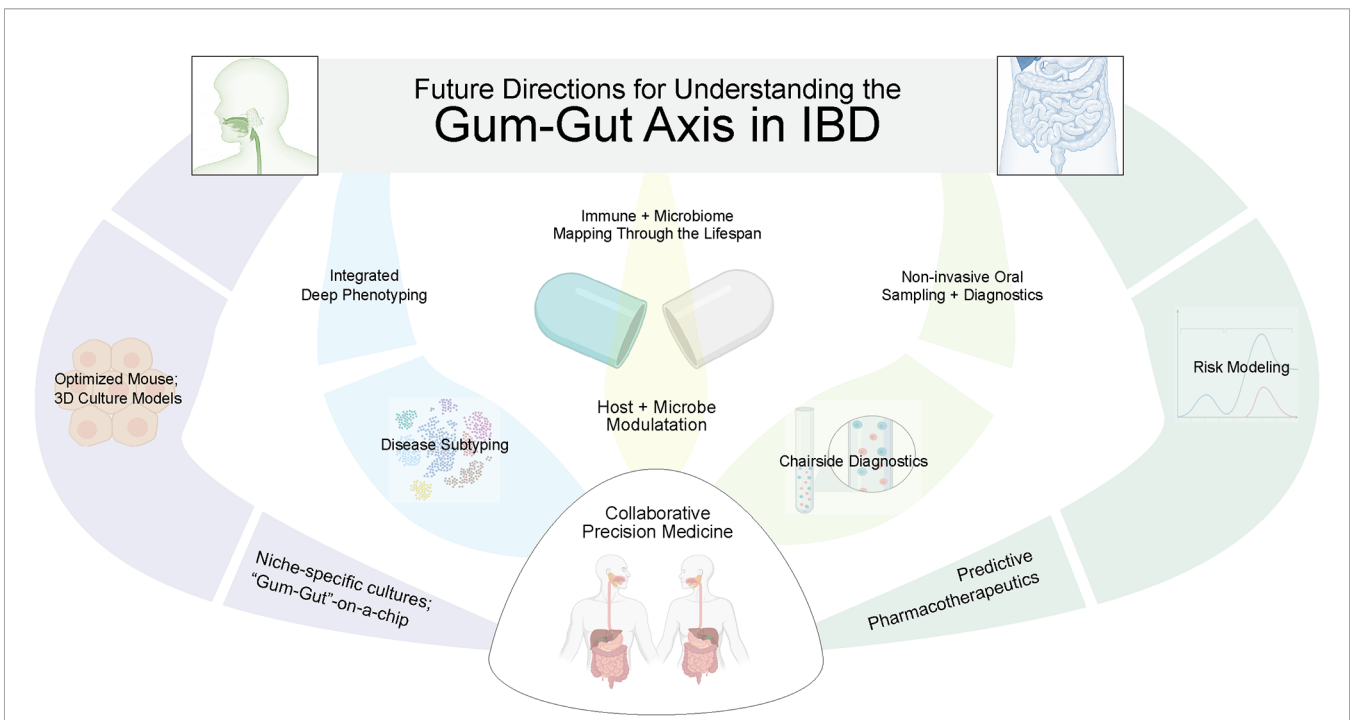

FIGURE 5 | Future directions to better elucidate the gum–gut axis. Due to the uniquely active and remittent inflammatory states of periodontal disease and inflammatory bowel disease, there is an exciting future for collaborative efforts between GI and oral health care providers to answer these questions. This will require biosampling both oral and intestinal sites to correlate dysbiosis and inflammatory changes across niches and across time, better modeling using *ex vivo* models, and better phenotyping of both diseases. In the future, predictive modeling and precision medical approaches are possible to treat both oral and GI diseases.

decade that originally collected nasal, oral, gut, skin, and vaginal microbiomes from a healthy cohort and provided valuable resources such as the Human Microbiome Project Data Coordination Center (202). As we begin to understand both the window of stable microbiomial establishment for the oral and gut microbiomes, we may be able to intervene to impact the health of these two sites for improved health later in life. For example, there are already several active clinical trials related to reestablishing a healthy neonatal gut microbiome (131). To justify the clinical implementation of this type of intervention will require *in vitro* 3D modeling (203), pre-clinical animal studies of host-microbiome interactions (204–207), interdisciplinary clinical research projects focused on longitudinal biobanking (208) for multiomics-informed approaches (199, 209, 210), biomarker discovery and validation, and improved risk modeling (211, 212). Ultimately, such work will lead to personalized therapeutics to target the **gum–gut** axis (213), sensitive and specific tools for early diagnosis of diseases in each site, and the exciting possibility of new biomarkers for risk stratifying a spectrum of oral and gastrointestinal diseases (214, 215).

AUTHOR CONTRIBUTIONS

Conceptualization: KB and AG. Investigation and data analysis: KB. Writing the original draft: KB. Writing, review and editing: KB and AG. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Braga AM, Squier CA. Ultrastructure of regenerating junctional epithelium in the monkey. *J Periodontol* (1980) 51(7):386–92. doi: 10.1902/jop.1980.51.7.386
2. Chen J, Jacox LA, Saldanha F, Sive H. Mouth development. *Wiley Interdiscip Rev: Dev Biol* (2017) 6(5):e275. doi: 10.1002/wdev.275
3. Nanci A, Ten CAR. *Ten Cate's Oral Histology: Development, Structure, and Function*. St. Louis, Mo: Mosby (2017).
4. Byrd KM, Piehl NC, Patel JH, Huh WJ, Sequeira I, Lough KJ, et al. Heterogeneity within Stratified Epithelial Stem Cell Populations Maintains the Oral Mucosa in Response to Physiological Stress. *Cell Stem Cell* (2019) 25(6):814–29.e816. doi: 10.1016/j.stem.2019.11.005
5. Regezi JA, Sciubba JJ, Jordan RC. *Oral pathology: clinical pathologic correlations. 6th Ed,* St. Louis: Elsevier Saunders (2016) 388.
6. Askitopoulou H, Nyktari V, Papaioannou A, Stefanakis G, Konsolaki E. The Origins of Oral Medicine in the Hippocratic Collected Works. *J Oral Pathol Med* (2017) 46(9):689–94. doi: 10.1111/jop.12615
7. Löe H. Periodontal diseases: a brief historical perspective. *Periodontol 2000* (1993) 2(1):7–12. doi: 10.1111/j.1600-0757.1993.tb00215.x
8. Baron JH. Inflammatory bowel disease up to 1932. *Mt Sinai J Med* (2000) 67 (3):174–89.
9. Donoff B, McDonough JE, Riedy CA. Integrating oral and general health care. *N Engl J Med* (2014) 371(24):2247–9. doi: 10.1056/NEJMp1410824
10. Rabiei S, Mohebbi SZ, Patja K, Virtanen JI. Physicians' knowledge of and adherence to improving oral health. *BMC Public Health* (2012) 12(1):1–9. doi: 10.1186/1471-2458-12-855
11. Elad S, Zadik Y, Caton JG, Epstein JB. Oral mucosal changes associated with primary diseases in other body systems. *Periodontol 2000* (2019) 80(1):28–48. doi: 10.1111/prd.12265
12. Squier CA, Kremer MJ. Biology of oral mucosa and esophagus. *JNCI Monogr* (2001) 2001(29):7–15. doi: 10.1093/oxfordjournals.jncimonographs.a003443
13. Muhvić-Urek M, Tomac-Stojmenović M, Mijandrušić-Sinčić B. Oral pathology in inflammatory bowel disease. *World J Gastroenterol* (2016) 22 (25):5655. doi: 10.3748/wjg.v22.i25.5655
14. Barnett ML. The oral-systemic disease connection: An update for the practicing dentist. *J Am Dental Assoc* (2006) 137:S5–6. doi: 10.14219/jada.archive.2006.0401
15. Genco RJ, Borgnakke WS. Risk factors for periodontal disease. *Periodontol 2000* (2013) 62(1):59–94. doi: 10.1111/j.1600-0757.2012.00457.x
16. Cianciola L, Park B, Bruck E, Mosovich L, Genco R. Prevalence of periodontal disease in insulin-dependent diabetes mellitus (juvenile diabetes). *J Am Dental Assoc* (1982) 104(5):653–60. doi: 10.14219/jada.archive.1982.0240
17. Beck J, Garcia R, Heiss G, Vokonas PS, Offenbacher S. Periodontal disease and cardiovascular disease. *J Periodontol* (1996) 67(10s):1123–37. doi: 10.1902/jop.1996.67.10s.1123
18. Offenbacher S, Katz V, Fertik G, Collins J, Boyd D, Maynor G, et al. Periodontal infection as a possible risk factor for preterm low birth weight. *J Periodontol* (1996) 67(10s):1103–13. doi: 10.1902/jop.1996.67.10s.1103
19. Cullinan MP, Seymour GJ. Periodontal disease and systemic illness: will the evidence ever be enough? *Periodontol 2000* (2013) 62(1):271–86. doi: 10.1111/prd.12007
20. Pihlstrom BL, Hodges JS, Michalowicz B, Wohlfahrt JC, Garcia RI. Promoting oral health care because of its possible effect on systemic disease is premature and may be misleading. *J Am Dent Assoc* (2018) 149 (6):401–3. doi: 10.1016/j.adaj.2018.03.030
21. Monsarrat P, Blaizot A, Kémoun P, Ravaud P, Nabet C, Sixou M, et al. Clinical research activity in periodontal medicine: a systematic mapping of trial registers. *J Clin Periodontol* (2016) 43(5):390–400. doi: 10.1111/jcpe.12534
22. Herrera D, Molina A, Buhlin K, Klinge B. Periodontal diseases and association with atherosclerotic disease. *Periodontol 2000* (2020) 83(1):66–89. doi: 10.1111/prd.12302
23. Bobetsis YA, Graziani F, Gürsoy M, Madianos PN. Periodontal disease and adverse pregnancy outcomes. *Periodontol 2000* (2020) 83(1):154–74. doi: 10.1111/prd.12294
24. Borgnakke WS, Ylöstalo PV, Taylor GW, Genco RJ. Effect of periodontal disease on diabetes: systematic review of epidemiologic observational evidence. *J Periodontol* (2013) 84:S135–52. doi: 10.1111/jcpe.12080
25. Rapone B, Corsalini M, Converti I, Loverro MT, Gnoni A, Trerotoli P, et al. Does Periodontal Inflammation Affect Type 1 Diabetes in Childhood and Adolescence? A Meta-Analysis. *Front Endocrinol* (2020) 11:278. doi: 10.3389/fendo.2020.00278
26. Jepsen S, Suvan J, Deschner J. The association of periodontal diseases with metabolic syndrome and obesity. *Periodontol 2000* (2020) 83(1):125–53. doi: 10.1111/prd.12326
27. Lira-Junior R, Figueredo CM. Periodontal and inflammatory bowel diseases: Is there evidence of complex pathogenic interactions? *World J Gastroenterol* (2016) 22(35):7963. doi: 10.3748/wjg.v22.i35.7963
28. Atarashi K, Suda W, Luo C, Kawaguchi T, Motoo I, Narushima S, et al. Ectopic colonization of oral bacteria in the intestine drives TH1 cell

induction and inflammation. *Science* (2017) 358(6361):359–65. doi: 10.1126/science.aan4526
29. Kitamoto S, Nagao-Kitamoto H, Jiao Y, Gillilland III MG, Hayashi A, Imai J, et al. The Intermucosal Connection between the Mouth and Gut in Commensal Pathobiont-Driven Colitis. *Cell* (2020) 182(2):447–62.e14. doi: 10.1016/j.cell.2020.05.048
30. Eke PI, Thornton-Evans GO, Wei L, Borgnakke WS, Dye BA, Genco RJ. Periodontitis in US adults: National health and nutrition examination survey 2009-2014. *J Am Dental Assoc* (2018) 149(7):576–88.e576. doi: 10.1016/j.adaj.2018.04.023
31. Kassebaum NJ, Bernabe E, Dahiya M, Bhandari B, Murray CJ, Marcenes W. Global burden of severe periodontitis in 1990-2010: a systematic review and meta-regression. *J Dental Res* (2014) 93(11):1045–53. doi: 10.1177/0022034514552491
32. Caton JG, Armitage G, Berglundh T, Chapple IL, Jepsen S, Kornman KS, et al. A new classification scheme for periodontal and peri-implant diseases and conditions–Introduction and key changes from the 1999 classification. *J Periodontol* (2018) 89:S1–8. doi: 10.1002/JPER.18-0157
33. Chapple IL, Mealey BL, Van Dyke TE, Bartold PM, Dommisch H, Eickholz P, et al. Periodontal health and gingival diseases and conditions on an intact and a reduced periodontium: Consensus report of workgroup 1 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Periodontol* (2018) 89:S74–84. doi: 10.1002/JPER.17-0719
34. Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH, et al. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Periodontol* (2018) 89:S173–82. doi: 10.1002/JPER.17-0721
35. Berezow AB, Darveau RP. Microbial shift and periodontitis. *Periodontol 2000* (2011) 55(1):36–47. doi: 10.1111/j.1600-0757.2010.00350.x
36. Abusleme L, Dupuy AK, Dutzan N, Silva N, Burleson JA, Strausbaugh LD, et al. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J* (2013) 7(5):1016–25. doi: 10.1038/ismej.2012.174
37. Kinane DF, Stathopoulou PG, Papapanou PN. Periodontal diseases. *Nat Rev Dis Primers* (2017) 3:17038. doi: 10.1038/nrdp.2017.38
38. Eke PI, Borgnakke WS, Genco RJ. Recent epidemiologic trends in periodontitis in the USA. *Periodontol 2000* (2020) 82(1):257–67. doi: 10.1111/prd.12323
39. Lauritano D, Boccalari E, Di Stasio D, Della Vella F, Carinci F, Lucchese A, et al. Prevalence of oral lesions and correlation with intestinal symptoms of inflammatory bowel disease: a systematic review. *Diagnostics* (2019) 9(3):77. doi: 10.3390/diagnostics9030077
40. GBD 2017 Inflammatory Bowel Disease Collaborators. The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Gastroenterol Hepatol* (2020) 5(1):17–30. doi: 10.1016/S2468-1253(19)30333-4
41. Haller CA, Markowitz J. IBD in children: lessons for adults. *Curr Gastroenterol Rep* (2007) 9(6):528–32. doi: 10.1007/s11894-007-0070-8
42. Kim HJ, Oh SH, Kim DY, Lee HS, Park SH, Yang SK, et al. Clinical Characteristics and Long-Term Outcomes of Paediatric Crohn's Disease: A Single-Centre Experience. *J Crohns Colitis* (2017) 11(2):157–64. doi: 10.1093/ecco-jcc/jjw146
43. Turpin W, Goethel A, Bedrani L, Croitoru MK. Determinants of IBD heritability: genes, bugs, and more. *Inflamm Bowel Dis* (2018) 24(6):1133–48. doi: 10.1093/ibd/izy085
44. Nibali L, Bayliss-Chapman J, Almofareh S, Zhou Y, Divaris K, Vieira A. What is the heritability of periodontitis? A systematic review. *J Dental Res* (2019) 98(6):632–41. doi: 10.1177/0022034519842510
45. Brandtzaeg P. Inflammatory bowel disease: clinics and pathology. Do inflammatory bowel disease and periodontal disease have similar immunopathogeneses? *Acta Odontol Scand* (2001) 59(4):235–43. doi: 10.1080/00016350152509265
46. Indriolo A, Greco S, Ravelli P, Fagiuoli S. What can we learn about biofilm/host interactions from the study of inflammatory bowel disease. *J Clin Periodontol* (2011) 38:36–43. doi: 10.1111/j.1600-051X.2010.01680.x
47. Zippi M, Corrado C, Pica R, Avallone EV, Cassieri C, De Nitto D, et al. Extraintestinal manifestations in a large series of Italian inflammatory bowel disease patients. *World J Gastroenterol* (2014) 20(46):17463. doi: 10.3748/wjg.v20.i46.17463
48. Greuter T, Bertoldo F, Rechner R, Straumann A, Biedermann L, Zeitz J, et al. Extraintestinal manifestations of pediatric inflammatory bowel disease: prevalence, presentation, and anti-TNF treatment. *J Pediatr Gastroenterol Nutr* (2017) 65(2):200–6. doi: 10.1097/MPG.0000000000001455
49. Pittock S, Drumm B, Fleming P, McDermott M, Imrie C, Flint S, et al. The oral cavity in Crohn's disease. *J Pediatr* (2001) 138(5):767–71. doi: 10.1067/mpd.2001.113008
50. Katz J, Shenkman A, Stavropoulos F, Melzer E. Oral signs and symptoms in relation to disease activity and site of involvement in patients with inflammatory bowel disease. *Oral Dis* (2003) 9(1):34–40. doi: 10.1034/j.1601-0825.2003.00879.x
51. Zbar AP, Ben-Horin S, Beer-Gabel M, Eliakim R. Oral Crohn's disease: Is it a separable disease from orofacial granulomatosis? A review. *J Crohn's Colitis* (2012) 6(2):135–42. doi: 10.1016/j.crohns.2011.07.001
52. Skrzat A, Olczak-Kowalczyk D, Turska-Szybka A. Crohn's disease should be considered in children with inflammatory oral lesions. *Acta Paediatr* (2017) 106(2):199–203. doi: 10.1111/apa.13686
53. Jose FA, Garnett EA, Vittinghoff E, Ferry GD, Winter HS, Baldassano RN, et al. Development of extraintestinal manifestations in pediatric patients with inflammatory bowel disease. *Inflamm Bowel Dis* (2009) 15(1):63–8. doi: 10.1002/ibd.20604
54. Chandan J, Thomas T. The impact of inflammatory bowel disease on oral health. *Br Dental J* (2017) 222(7):549–53. doi: 10.1038/sj.bdj.2017.318
55. Katsanos K, Torres J, Roda G, Brygo A, Delaporte E, Colombel JF. Non-malignant oral manifestations in inflammatory bowel diseases. *Alimentary Pharmacol Ther* (2015) 42(1):40–60. doi: 10.1111/apt.13217
56. Ojha J, Cohen DM, Islam NM, Stewart CM, Katz J, Bhattacharyya I. Gingival involvement in Crohn disease. *J Am Dental Assoc* (2007) 138(12):1574–81. doi: 10.14219/jada.archive.2007.0106
57. Plauth M, Jenss H, Meyle J. Oral manifestations of Crohn's disease. An analysis of 79 cases. *J Clin Gastroenterol* (1991) 13(1):29–37. doi: 10.1097/00004836-199102000-00008
58. Eckel A, Lee D, Deutsch G, Maxin A, Oda D. Oral manifestations as the first presenting sign of Crohn's disease in a pediatric patient. *J Clin Exp Dent* (2017) 9(7):e934. doi: 10.4317/jced.53914
59. NP Lang, Lindhe J eds. *Clinical Periodontology and Implant Dentistry*. New York, NY, USA: John Wiley & Sons (2015).
60. Lang NP, Bartold PM. Periodontal health. *J Periodontol* (2018) 89:S9–S16. doi: 10.1002/JPER.16-0517
61. Löe H, Theilade E, Jensen SB. Experimental Gingivitis in Man. *J Periodontol* (1965) 36:177–87. doi: 10.1902/jop.1965.36.3.177
62. Page RC, Schroeder HE. Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest* (1976) 34(3):235–49.
63. Trombelli L, Farina R, Silva CO, Tatakis DN. Plaque-induced gingivitis: Case definition and diagnostic considerations. *J Clin Periodontol* (2018) 45:S44–67. doi: 10.1111/jcpe.12939
64. Hugoson A, Koch G, Rylander H. Prevalence and distribution of gingivitis-periodontitis in children and adolescents. Epidemiological data as a base for risk group selection. *Swed Dent J* (1981) 5(3):91–103.
65. Lindhe J, Rylander H. Experimental gingivitis in young dogs. *Eur J Oral Sci* (1975) 83(6):314–26. doi: 10.1111/j.1600-0722.1975.tb00444.x
66. Zemouri C, Jakubovics NS, Crielaard W, Zaura E, Dodds M, Schelkle B, et al. Resistance and resilience to experimental gingivitis: a systematic scoping review. *BMC Oral Health* (2019) 19(1):212. doi: 10.1186/s12903-019-0889-z
67. Schincaglia G, Hong B, Rosania A, Barasz J, Thompson A, Sobue T, et al. Clinical, immune, and microbiome traits of gingivitis and peri-implant mucositis. *J Dental Res* (2017) 96(1):47–55. doi: 10.1177/0022034516668847
68. Mira A, Simon-Soro A, Curtis M. Role of microbial communities in the pathogenesis of periodontal diseases and caries. *J Clin Periodontol* (2017) 44:S23–38. doi: 10.1111/jcpe.12671
69. Lim ML, Wallace MR. Infectious diarrhea in history. *Infect Dis Clinics North Am* (2004) 18(2):261. doi: 10.1016/j.idc.2004.01.006
70. Mulder DJ, Noble AJ, Justinich CJ, Duffin JM. A tale of two diseases: the history of inflammatory bowel disease. *J Crohn's Colitis* (2014) 8(5):341–8. doi: 10.1016/j.crohns.2013.09.009

71. Merritt AH. A brief history of periodontology. *J Dental Res* (1921) 3(4): cxlix–clxi. doi: 10.1177/00220345210030040707
72. Sircus W, Church R, Kelleher J. Recurrent aphthous ulceration of the mouth; a study of the natural history, aetiology, and treatment. *Q J Med* (1957) 26 (102):235–49.
73. Dudeney TP. Crohn's disease of the mouth. *Proc R Soc Med* (1969) 62 (12):1237. doi: 10.1177/003591576906201218
74. Croft CB, Wilkinson AR. Ulceration of the mouth, pharynx, and larynx in Crohn's disease of the intestine. *Br J Surg* (1972) 59(4):249–52. doi: 10.1002/bjs.1800590402
75. Stankler L, Ewen SW, Kerr NW. Crohn's disease of the mouth. *Br J Dermatol* (1972) 87(5):501–4. doi: 10.1111/j.1365-2133.1972.tb01599.x
76. Asquith P, Thompson RA, Cooke WT. Oral manifestations of Crohn's disease. *Gut* (1975) 16(4):249–54. doi: 10.1136/gut.16.4.249
77. Snyder M, Cawson R. Oral changes in Crohn's disease. *J Oral Surg* (1976) 34 (7):594–9.
78. Schiller KF, Golding PL, Peebles RA, Whitehead R. Crohn's disease of the mouth and lips. *Gut* (1971) 12(10):864–5.
79. Ellis JP. Crohn's disease with mouth involvement. *Proc R Soc Med* (1972) 65 (12):1080. doi: 10.1177/003591577206501211
80. Bottomley WK, Giorgini GL, Julienne CH. Oral extension of regional enteritis (Crohn's disease). Report of a case. *Oral Surg Oral Med Oral Pathol* (1972) 34(3):417–20. doi: 10.1016/0030-4220(72)90317-9
81. Bernstein ML, McDonald JS. Oral lesions in Crohn's disease: report of two cases and update of the literature. *Oral Surg Oral Med Oral Pathol* (1978) 46 (2):234–45. doi: 10.1016/0030-4220(78)90198-6
82. Sachar D, Auslander M. Missing pieces in the puzzle of Crohn's disease. *Gastroenterology* (1978) 75(4):745. doi: 10.1016/S0016-5085(19)31718-4
83. Lamster IB, Rodrick ML, Sonis ST, Falchuk ZM. An analysis of peripheral blood and salivary polymorphonuclear leukocyte function, circulating immune complex levels and oral status in patients with inflammatory bowel disease. *J Periodontol* (1982) 53(4):231–8. doi: 10.1902/jop.1982.53.4.231
84. Engel LD, Pasquinelli KL, Leone SA, Moncla BJ, Nielson KD, Rabinovitch PS. Abnormal lymphocyte profiles and leukotriene B4 status in a patient with Crohn's disease and severe periodontitis. *J Periodontol* (1988) 59 (12):841–7. doi: 10.1902/jop.1988.59.12.841
85. Koutsochristou V, Zellos A, Dimakou K, Panayotou I, Siahanidou S, Roma-Giannikou E, et al. Dental Caries and Periodontal Disease in Children and Adolescents with Inflammatory Bowel Disease: A Case–Control Study. *Inflamm Bowel Dis* (2015) 21(8):1839–46. doi: 10.1097/MIB.0000000000000452
86. Flemmig TF, Shanahan F, Miyasaki KT. Prevalence and severity of periodontal disease in patients with inflammatory bowel disease. *J Clin Periodontol* (1991) 18(9):690–7. doi: 10.1111/j.1600-051X.1991.tb00111.x
87. Grössner-Schreiber B, Fetter T, Hedderich J, Kocher T, Schreiber S, Jepsen S. Prevalence of dental caries and periodontal disease in patients with inflammatory bowel disease: a case–control study. *J Clin Periodontol* (2006) 33(7):478–84. doi: 10.1111/j.1600-051X.2006.00942.x
88. Brito F, Barros FCD, Zaltman C, Pugas Carvalho AT, de Vasconcellos Carneiro AJ, Fischer RG, et al. Prevalence of periodontitis and DMFT index in patients with Crohn's disease and ulcerative colitis. *J Clin Periodontol* (2008) 35(6):555–60. doi: 10.1111/j.1600-051X.2008.01231.x
89. Habashneh R, Khader Y, Alhumouz M, Jadallah K, Ajlouni Y. The association between inflammatory bowel disease and periodontitis among Jordanians: a case–control study. *J Periodontal Res* (2012) 47(3):293–8. doi: 10.1111/j.1600-0765.2011.01431.x
90. Vavricka SR, Manser CN, Hediger S, Vögelin M, Scharl M, Biedermann L, et al. Periodontitis and Gingivitis in Inflammatory Bowel Disease: A Case–Control Study. *Inflamm Bowel Dis* (2013) 19(13):2768–77. doi: 10.1097/01.MIB.0000438356.84263.3b
91. She Y-y, Kong X-B, Ge Y-P, Liu Z-Y, Chen J-Y, Jiang J-W, et al. Periodontitis and inflammatory bowel disease: a meta-analysis. *BMC Oral Health* (2020) 20(1):1–11. doi: 10.1186/s12903-020-1053-5
92. Papageorgiou SN, Hagner M, Nogueira AV, Franke A, Jager A, Deschner J. Inflammatory bowel disease and oral health: systematic review and a meta-analysis. *J Clin Periodontol* (2017) 44(4):382–93. doi: 10.1111/jcpe.12698
93. Ten Cate AR. The role of epithelium in the development, structure and function of the tissues of tooth support. *Oral Dis* (1996) 2(1):55–62. doi: 10.1111/j.1601-0825.1996.tb00204.x
94. Bosshardt DD, Lang NP. The junctional epithelium: from health to disease. *J Dental Res* (2005) 84(1):9–20. doi: 10.1177/154405910508400102
95. Dutzan N, Abusleme L, Bridgeman H, Greenwell-Wild T, Zangerle-Murray T, Fife ME, et al. On-going Mechanical Damage from Mastication Drives Homeostatic Th17 Cell Responses at the Oral Barrier. *Immunity* (2017) 46 (1):133–47. doi: 10.1016/j.immuni.2016.12.010
96. Curtis MA, Diaz PI, Van Dyke TE. The role of the microbiota in periodontal disease. *Periodontol 2000* (2020) 83(1):14–25. doi: 10.1111/prd.12296
97. Moskow BS, Poison AM. Histologic studies on the extension of the inflammatory infiltrate in human periodontitis. *J Clin Periodontol* (1991) 18(7):534–42. doi: 10.1111/j.1600-051X.1991.tb00086.x
98. Dutzan N, Konkel JE, Greenwell-Wild T, Moutsopoulos NM. Characterization of the human immune cell network at the gingival barrier. *Mucosal Immunol* (2016) 9(5):1163–72. doi: 10.1038/mi.2015.136
99. Presland RB, Dale BA. Epithelial structural proteins of the skin and oral cavity: function in health and disease. *Crit Rev Oral Biol Med* (2000) 11 (4):383–408. doi: 10.1177/10454411000110040101
100. Dale BA. Periodontal epithelium: a newly recognized role in health and disease. *Periodontol 2000* (2002) 30:70–8. doi: 10.1034/j.1600-0757.2002.03007.x
101. Fujita T, Yoshimoto T, Kajiya M, Ouhara K, Matsuda S, Takemura T, et al. Regulation of defensive function on gingival epithelial cells can prevent periodontal disease. *Jpn Dental Sci Rev* (2018) 54(2):66–75. doi: 10.1016/j.jdsr.2017.11.003
102. Moutsopoulos NM, Konkel JE. Tissue-Specific Immunity at the Oral Mucosal Barrier. *Trends Immunol* (2018) 39(4):276–87. doi: 10.1016/j.it.2017.08.005
103. Huang N, Perez P, Kato T, Mikami Y, Okuda K, Gilmore RC, et al. Integrated Single-Cell Atlases Reveal an Oral SARS-CoV-2 Infection and Transmission Axis. *medRxiv* [Preprint] (2020). doi: 10.1101/2020.10.26.20219089. 2020.2010.2026.20219089.
104. Caetano AJ, Yianni V, Volponi A, Booth V, D'Agostino EM, Sharpe P. Defining human mesenchymal and epithelial heterogeneity in response to oral inflammatory disease. *Elife* (2021) 10:e62810. doi: 10.7554/eLife.62810
105. Nishida M, Grossi SG, Dunford RG, Ho AW, Trevisan M, Genco RJ. Dietary vitamin C and the risk for periodontal disease. *J Periodontol* (2000) 71 (8):1215–23. doi: 10.1902/jop.2000.71.8.1215
106. Hou JK, Abraham B, El-Serag H. Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature. *Am J Gastroenterol* (2011) 106(4):563–73. doi: 10.1038/ajg.2011.44
107. Yan H, Wang H, Zhang X, Li X, Yu J. Ascorbic acid ameliorates oxidative stress and inflammation in dextran sulfate sodium-induced ulcerative colitis in mice. *Int J Clin Exp Med* (2015) 8(11):20245–53.
108. Najeeb S, Zafar MS, Khurshid Z, Zohaib S, Almas K. The role of nutrition in periodontal health: an update. *Nutrients* (2016) 8(9):530. doi: 10.3390/nu8090530
109. Lamster I, Sonis S, Hannigan A, Kolodkin A. An association between Crohn's disease, periodontal disease and enhanced neutrophil function. *J Periodontol* (1978) 49(9):475–9. doi: 10.1902/jop.1978.49.9.475
110. Said HS, Suda W, Nakagome S, Chinen H, Oshima K, Kim S, et al. Dysbiosis of salivary microbiota in inflammatory bowel disease and its association with oral immunological biomarkers. *DNA Res* (2013) 21(1):15–25. doi: 10.1093/dnares/dst037
111. Szczeklik K, Owczarek D, Pytko-Polończyk J, Kęsek B, Mach TH. Proinflammatory cytokines in the saliva of patients with active and nonactive Crohn's. *Pol Arch Med Wewn* (2012) 122(5):200–8. doi: 10.20452/pamw.1256
112. Figueredo CM, Martins AP, Lira-Junior R, Menegat JB, Carvalho AT, Fischer RG, et al. Activity of inflammatory bowel disease influences the expression of cytokines in gingival tissue. *Cytokine* (2017) 95:1–6. doi: 10.1016/j.cyto.2017.01.016
113. Damen GM, Hol J, de Ruiter L, Bouquet J, Sinaasappel M, van der Woude J, et al. Chemokine production by buccal epithelium as a distinctive feature of pediatric Crohn disease. *J Pediatr Gastroenterol Nutr* (2006) 42(2):142–9. doi: 10.1097/01.mpg.0000189336.70021.8a

114. Menegat JSB, Lira-Junior R, Siqueira MA, Brito F, Carvalho AT, Fischer RG, et al. Cytokine expression in gingival and intestinal tissues of patients with periodontitis and inflammatory bowel disease: An exploratory study. *Arch Oral Biol* (2016) 66:141–6. doi: 10.1016/j.archoralbio.2016.02.018
115. Van Dyke T, Dowell V, Offenbacher S, Snyder W, Hersh T. Potential role of microorganisms isolated from periodontal lesions in the pathogenesis of inflammatory bowel disease. *Infect Immun* (1986) 53(3):671–7. doi: 10.1128/IAI.53.3.671-677.1986
116. Kaakoush NO, Mitchell HM. Campylobacter concisus–a new player in intestinal disease. *Front Cell Infect Microbiol* (2012) 2:4. doi: 10.3389/fcimb.2012.00004
117. Liu F, Ma R, Wang Y, Zhang L. The clinical importance of Campylobacter concisus and other human hosted Campylobacter species. *Front Cell Infect Microbiol* (2018) 8:243. doi: 10.3389/fcimb.2018.00243
118. Graves D, Corrêa J, Silva T. The oral microbiota is modified by systemic diseases. *J Dental Res* (2019) 98(2):148–56. doi: 10.1177/0022034518805739
119. Rautava J, Pinnell LJ, Vong L, Akseer N, Assa A, Sherman PM. Oral microbiome composition changes in mouse models of colitis. *J Gastroenterol Hepatol* (2015) 30(3):521–7. doi: 10.1111/jgh.12713
120. Brakenhoff LK, van der Heijde DM, Hommes DW, Huizinga TW, Fidder HH. The joint—gut axis in inflammatory bowel diseases. *J Crohn's Colitis* (2010) 4(3):257–68. doi: 10.1016/j.crohns.2009.11.005
121. Vavricka SR, Schoepfer A, Scharl M, Lakatos PL, Navarini A, Rogler G. Extraintestinal manifestations of inflammatory bowel disease. *Inflamm Bowel Dis* (2015) 21(8):1982–92. doi: 10.1097/MIB.0000000000000392
122. Docktor MJ, Paster BJ, Abramowicz S, Ingram J, Wang YE, Correll M, et al. Alterations in diversity of the oral microbiome in pediatric inflammatory bowel disease. *Inflamm Bowel Dis* (2012) 18(5):935–42. doi: 10.1002/ibd.21874
123. Kelsen J, Bittinger K, Pauly-Hubbard H, Posivak L, Grunberg S, Baldassano R, et al. Alterations of the subgingival microbiota in pediatric Crohn's disease studied longitudinally in discovery and validation cohorts. *Inflamm Bowel Dis* (2015) 21(12):2797–805. doi: 10.1097/MIB.0000000000000557
124. Brito F, Zaltman C, Carvalho AT, Fischer RG, Persson R, Gustafsson A, et al. Subgingival microflora in inflammatory bowel disease patients with untreated periodontitis. *Eur J Gastroenterol Hepatol* (2013) 25(2):239–45. doi: 10.1097/MEG.0b013e32835a2b70
125. Xun Z, Zhang Q, Xu T, Chen N, Chen F. Dysbiosis and ecotypes of the salivary microbiome associated with inflammatory bowel diseases and the assistance in diagnosis of diseases using oral bacterial profiles. *Front Microbiol* (2018) 9:1136. doi: 10.3389/fmicb.2018.01136
126. Fourie NH, Wang D, Abey SK, Sherwin LB, Joseph PV, Rahim-Williams B, et al. The microbiome of the oral mucosa in irritable bowel syndrome. *Gut Microbes* (2016) 7(4):286–301. doi: 10.1080/19490976.2016.1162363
127. Gensollen T, Iyer SS, Kasper DL, Blumberg RS. How colonization by microbiota in early life shapes the immune system. *Science* (2016) 352 (6285):539–44. doi: 10.1126/science.aad9378
128. Ramanan D, Sefik E, Galván-Peña S, Wu M, Yang L, Yang Z, et al. An immunologic mode of multigenerational transmission governs a gut Treg setpoint. *Cell* (2020) 181(6):1276–90.e13. doi: 10.1016/j.cell.2020.04.030
129. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu W-H, et al. The human oral microbiome. *J Bacteriol* (2010) 192(19):5002–17. doi: 10.1128/JB.00542-10
130. Xiao J, Fiscella KA, Gill SR. Oral microbiome: possible harbinger for children's health. *Int J Oral Sci* (2020) 12(1):1–13. doi: 10.1038/s41368-020-0082-x
131. Dominguez-Bello MG, Godoy-Vitorino F, Knight R, Blaser MJ. Role of the microbiome in human development. *Gut* (2019) 68(6):1108–14. doi: 10.1136/gutjnl-2018-317503
132. Gest H. The discovery of microorganisms by Robert Hooke and Antoni Van Leeuwenhoek, fellows of the Royal Society. *Notes Rec R Soc London* (2004) 58 (2):187–201. doi: 10.1098/rsnr.2004.0055
133. Escapa I, Chen T, Huang Y, Gajare P, Dewhirst F, Lemon K. New Insights into Human Nostril Microbiome from the Expanded Human Oral Microbiome Database (eHOMD): a Resource for the Microbiome of the Human Aerodigestive Tract. *mSystems* (2018) 3(*6*):e00187–18. doi: 10.1128/mSystems.00187-18
134. Gao L, Xu T, Huang G, Jiang S, Gu Y, Chen F. Oral microbiomes: more and more importance in oral cavity and whole body. *Protein Cell* (2018) 9 (5):488–500. doi: 10.1007/s13238-018-0548-1
135. Aleti G, Baker JL, Tang X, Alvarez R, Dinis M, Tran NC, et al. Identification of the bacterial biosynthetic gene clusters of the oral microbiome illuminates the unexplored social language of bacteria during health and disease. *MBio* (2019) 10(2):e00321–19. doi: 10.1128/mBio.00321-19
136. Balachandran M, Cross KL, Podar M. Single-cell genomics and the oral microbiome. *J Dental Res* (2020) 99(6):613–20. doi: 10.1177/0022034520907380
137. Lamont RJ, Koo H, Hajishengallis G. The oral microbiota: dynamic communities and host interactions. *Nat Rev Microbiol* (2018) 16(12):745–59. doi: 10.1038/s41579-018-0089-x
138. Segata N, Haake SK, Mannon P, Lemon KP, Waldron L, Gevers D, et al. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. *Genome Biol* (2012) 13(6): R42. doi: 10.1186/gb-2012-13-6-r42
139. Maki KA, Kazmi N, Barb JJ, Ames N. The Oral and Gut Bacterial Microbiomes: Similarities, Differences, and Connections. *Biol Res For Nurs* (2021) 23(1):7–20 doi: 10.1177/1099800420941606
140. Kilian M, Chapple I, Hannig M, Marsh P, Meuric V, Pedersen A, et al. The oral microbiome–an update for oral healthcare professionals. *Br Dental J* (2016) 221(10):657. doi: 10.1038/sj.bdj.2016.865
141. Waggoner-Fountain LA, Whit Walker M, Hollis RJ, Pfaller MA, Ferguson JE, Wenzel RP, et al. Vertical and horizontal transmission of unique Candida species to premature newborns. *Clin Infect Dis* (1996) 22(5):803–8. doi: 10.1093/clinids/22.5.803
142. Bliss JM, Basavegowda KP, Watson WJ, Sheikh AU, Ryan RM. Vertical and horizontal transmission of Candida albicans in very low birth weight infants using DNA fingerprinting techniques. *Pediatr Infect Dis J* (2008) 27(3):231–5. doi: 10.1097/INF.0b013e31815bb69d
143. Brito IL, Gurry T, Zhao S, Huang K, Young SK, Shea TP, et al. Transmission of human-associated microbiota along family and social networks. *Nat Microbiol* (2019) 4(6):964–71. doi: 10.1038/s41564-019-0409-6
144. Payne M, Hashim A, Alsam A, Joseph S, Aduse-Opoku J, Wade W, et al. Horizontal and Vertical Transfer of Oral Microbial Dysbiosis and Periodontal Disease. *J Dental Res* (2019) 98(13):1503–10. doi: 10.1177/0022034519877150
145. Xu X, He J, Xue J, Wang Y, Li K, Zhang K, et al. Oral cavity contains distinct niches with dynamic microbial communities. *Environ Microbiol* (2015) 17 (3):699–710. doi: 10.1111/1462-2920.12502
146. Gensollen T, Blumberg RS. Correlation between early-life regulation of the immune system by microbiota and allergy development. *J Allergy Clin Immunol* (2017) 139(4):1084–91. doi: 10.1016/j.jaci.2017.02.011
147. Constantinides MG, Link VM, Tamoutounour S, Wong AC, Perez-Chaparro PJ, Han S-J, et al. MAIT cells are imprinted by the microbiota in early life and promote tissue repair. *Science* (2019) 366(6464):eaax6624. doi: 10.1126/science.aax6624
148. Al Nabhani Z, Eberl G. Imprinting of the immune system by the microbiota early in life. *Mucosal Immunol* (2020) 13(2):183–9. doi: 10.1038/s41385-020-0257-y
149. Perez-Muñoz ME, Arrieta M-C, Ramer-Tait AE, Walter J. A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: implications for research on the pioneer infant microbiome. *Microbiome* (2017) 5(1):48. doi: 10.1186/s40168-017-0268-4
150. Stinson LF, Boyce MC, Payne MS, Keelan JA. The not-so-sterile womb: evidence that the human fetus is exposed to bacteria prior to birth. *Front Microbiol* (2019) 10:1124. doi: 10.3389/fmicb.2019.01124
151. Rackaityte E, Halkias J, Fukui E, Mendoza V, Hayzelden C, Crawford E, et al. Viable bacterial colonization is highly limited in the human intestine in utero. *Nat Med* (2020) 26(4):599–607. doi: 10.1038/s41591-020-0761-3
152. Ding T, Schloss PD. Dynamics and associations of microbial community types across the human body. *Nature* (2014) 509(7500):357–60. doi: 10.1038/nature13178
153. Rodríguez JM, Murphy K, Stanton C, Ross RP, Kober OI, Juge N, et al. The composition of the gut microbiota throughout life, with an emphasis on early life. *Microbial Ecol Health Dis* (2015) 26(1):26050. doi: 10.3402/mehd.v26.26050

154. Stras SF, Werner L, Toothaker JM, Olaloye OO, Oldham AL, McCourt CC, et al. Maturation of the human intestinal immune system occurs early in fetal development. *Dev Cell* (2019) 51(3):357–73.e355. doi: 10.1016/j.devcel.2019.09.008
155. Ruiz L, Delgado S, Ruas-Madiedo P, Sánchez B, Margolles A. Bifidobacteria and their molecular communication with the immune system. *Front Microbiol* (2017) 8:2345. doi: 10.3389/fmicb.2017.02345
156. Derrien M, van Passel MW, van de Bovenkamp JH, Schipper R, de Vos W, Dekker J. Mucin-bacterial interactions in the human oral cavity and digestive tract. *Gut Microbes* (2010) 1(4):254–68. doi: 10.4161/gmic.1.4.12778
157. Toda K, Hisata K, Satoh T, Katsumata N, Odamaki T, Mitsuyama E, et al. Neonatal oral fluid as a transmission route for bifidobacteria to the infant gut immediately after birth. *Sci Rep* (2019) 9(1):1–9. doi: 10.1038/s41598-019-45198-9
158. Weström B, Arévalo Sureda E, Pierzynowska K, Pierzynowski SG, Pérez-Cano F-J. The Immature Gut Barrier and Its Importance in Establishing Immunity in Newborn Mammals. *Front Immunol* (2020) 11:1153. doi: 10.3389/fimmu.2020.01153
159. Abeles SR, Robles-Sikisaka R, Ly M, Lum AG, Salzman J, Boehm TK, et al. Human oral viruses are personal, persistent and gender-consistent. *ISME J* (2014) 8(9):1753–67. doi: 10.1038/ismej.2014.31
160. Wilbert SA, Welch JLM, Borisy GG. Spatial ecology of the human tongue dorsum microbiome. *Cell Rep* (2020) 30(12):4003–15.e4003. doi: 10.1016/j.celrep.2020.02.097
161. Mark Welch JL, Dewhirst FE, Borisy GG. Biogeography of the Oral microbiome: the site-specialist hypothesis. *Annu Rev Microbiol* (2019) 73:335–58. doi: 10.1146/annurev-micro-090817-062503
162. Proctor DM, Fukuyama JA, Loomer PM, Armitage GC, Lee SA, Davis NM, et al. A spatial gradient of bacterial diversity in the human oral cavity shaped by salivary flow. *Nat Commun* (2018) 9(1):681. doi: 10.1038/s41467-018-02900-1
163. Proctor DM, Relman DA. The landscape ecology and microbiota of the human nose, mouth, and throat. *Cell Host Microbe* (2017) 21(4):421–32. doi: 10.1016/j.chom.2017.03.011
164. Huang S, Haiminen N, Carrieri A-P, Hu R, Jiang L, Parida L, et al. Human Skin, Oral, and Gut Microbiomes Predict Chronological Age. *mSystems* (2020) 5(1):e00630–19. doi: 10.1128/mSystems.00630-19
165. Freire M, Moustafa A, Harkins DM, Torralba MG, Zhang Y, Leong P, et al. Longitudinal Study of oral Microbiome Variation in twins. *Sci Rep* (2020) 10(1):1–10. doi: 10.1038/s41598-020-64747-1
166. Holgerson PL, Esberg A, Sjödin A, West CE, Johansson I. A longitudinal study of the development of the saliva microbiome in infants 2 days to 5 years compared to the microbiome in adolescents. *Sci Rep* (2020) 10(1):1–14. doi: 10.1038/s41598-020-66658-7
167. Mason MR, Chambers S, Dabdoub SM, Thikkurissy S, Kumar PS. Characterizing oral microbial communities across dentition states and colonization niches. *Microbiome* (2018) 6(1):67. doi: 10.1186/s40168-018-0443-2
168. Holgerson PL, Öhman C, Rönnlund A, Johansson I. Maturation of oral microbiota in children with or without dental caries. *PLoS One* (2015) 10(5):e0128534. doi: 10.1371/journal.pone.0128534
169. Tuominen H, Collado MC, Rautava J, Syrjänen S, Rautava S. Composition and maternal origin of the neonatal oral cavity microbiota. *J Oral Microbiol* (2019) 11(1):1663084. doi: 10.1080/20002297.2019.1663084
170. Uchiyama K, Haruyama Y, Shiraishi H, Katahira K, Abukawa D, Ishige T, et al. Association between Passive Smoking from the Mother and Pediatric Crohn's Disease: A Japanese Multicenter Study. *Int J Environ Res Public Health* (2020) 17(8):2926. doi: 10.3390/ijerph17082926
171. Kenney EB, Ash MMJr. Oxidation reduction potential of developing plaque, periodontal pockets and gingival sulci. *J Periodontol* (1969) 40(11):630–3. doi: 10.1902/jop.1969.40.11.630
172. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, et al. Bacterial diversity in human subgingival plaque. *J Bacteriol* (2001) 183(12):3770–83. doi: 10.1128/JB.183.12.3770-3783.2001
173. Eggert F, Drewell L, Bigelow J, Speck J, Goldner M. The pH of gingival crevices and periodontal pockets in children, teenagers and adults. *Arch Oral Biol* (1991) 36(3):233–8. doi: 10.1016/0003-9969(91)90091-8
174. Marsh PD, Bradshaw DJ. Physiological approaches to the control of oral biofilms. *Adv Dental Res* (1997) 11(1):176–85. doi: 10.1177/08959374970110010901
175. Asikainen S, Doğan B, Turgut Z, Paster BJ, Bodur A, Oscarsson J. Specified species in gingival crevicular fluid predict bacterial diversity. *PLoS One* (2010) 5(10):e13589. doi: 10.1371/journal.pone.0013589
176. Zhou J, Yao Y, Jiao K, Zhang J, Zheng X, Wu F, et al. Relationship between gingival crevicular fluid microbiota and cytokine profile in periodontal host homeostasis. *Front Microbiol* (2017) 8:2144. doi: 10.3389/fmicb.2017.02144
177. Liu S, Wang Y, Zhao L, Sun X, Feng Q. Microbiome succession with increasing age in three oral sites. *Aging* (2020) 12(9):7874. doi: 10.18632/aging.103108
178. Carpenter G. Salivary factors that maintain the normal oral commensal microflora. *J Dental Res* (2020) 99(6):644–9. doi: 10.1177/0022034520915486
179. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol* (2015) 15(1):30–44. doi: 10.1038/nri3785
180. Lourenço TGB, Spencer SJ, Alm EJ, Colombo APV. Defining the gut microbiota in individuals with periodontal diseases: an exploratory study. *J Oral Microbiol* (2018) 10(1):1487741. doi: 10.1080/20002297.2018.1487741
181. Mall AS, Habte H, Mthembu Y, Peacocke J, De Beer C. Mucus and Mucins: do they have a role in the inhibition of the human immunodeficiency virus? *Virol J* (2017) 14(1):1–14. doi: 10.1186/s12985-017-0855-9
182. Olsen I, Yamazaki K. Can oral bacteria affect the microbiome of the gut? *J Oral Microbiol* (2019) 11(1):1586422. doi: 10.1080/20002297.2019.1586422
183. Arimatsu K, Yamada H, Miyazawa H, Minagawa T, Nakajima M, Ryder MI, et al. Oral pathobiont induces systemic inflammation and metabolic changes associated with alteration of gut microbiota. *Sci Rep* (2014) 4:4828. doi: 10.1038/srep04828
184. Kitamoto S, Nagao-Kitamoto H, Hein R, Schmidt T, Kamada N. The Bacterial Connection between the Oral Cavity and the Gut Diseases. *J Dental Res* (2020) 99(9):1021–9. doi: 10.1177/0022034520924633
185. Nardone G, Compare D, Rocco A. A microbiota-centric view of diseases of the upper gastrointestinal tract. *Lancet Gastroenterol Hepatol* (2017) 2(4):298–312. doi: 10.1016/S2468-1253(16)30108-X
186. Pei Z, Bini EJ, Yang L, Zhou M, Francois F, Blaser MJ. Bacterial biota in the human distal esophagus. *Proc Natl Acad Sci* (2004) 101(12):4250–5. doi: 10.1073/pnas.0306398101
187. Muhlebach MS, Zorn BT, Esther CR, Hatch JE, Murray CP, Turkovic L, et al. Initial acquisition and succession of the cystic fibrosis lung microbiome is associated with disease progression in infants and preschool children. *PLoS Pathog* (2018) 14(1):e1006798. doi: 10.1371/journal.ppat.1006798
188. Gevers D, Kugathasan S, Denson LA, Vázquez-Baeza Y, Van Treuren W, Ren B, et al. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe* (2014) 15(3):382–92. doi: 10.1016/j.chom.2014.02.005
189. Yachida S, Mizutani S, Shiroma H, Shiba S, Nakajima T, Sakamoto T, et al. Metagenomic and metabolomic analyses reveal distinct stage-specific phenotypes of the gut microbiota in colorectal cancer. *Nat Med* (2019) 25(6):968–76. doi: 10.1038/s41591-019-0458-7
190. Schmidt TS, Hayward MR, Coelho LP, Li SS, Costea PI, Voigt AY, et al. Extensive transmission of microbes along the gastrointestinal tract. *eLife* (2019) 8:e42693. doi: 10.7554/eLife.42693
191. Li B, Ge Y, Cheng L, Zeng B, Yu J, Peng X, et al. Oral bacteria colonize and compete with gut microbiota in gnotobiotic mice. *Int J Oral Sci* (2019) 11(1):1–9. doi: 10.1038/s41368-018-0043-9
192. Zeller G, Tap J, Voigt AY, Sunagawa S, Kultima JR, Costea PI, et al. Potential of fecal microbiota for early-stage detection of colorectal cancer. *Mol Syst Biol* (2014) 10(11):766. doi: 10.15252/msb.20145645
193. Dickson RP, Erb-Downward JR, Freeman CM, McCloskey L, Falkowski NR, Huffnagle GB, et al. Bacterial topography of the healthy human lower respiratory tract. *MBio* (2017) 8(1):e02287–02216. doi: 10.1128/mBio.02287-16
194. Dinakaran V, Mandape SN, Shuba K, Pratap S, Sakhare SS, Tabatabai MA, et al. Identification of specific oral and gut pathogens in full thickness colon of colitis patients: implications for colon motility. *Front Microbiol* (2019) 9:3220. doi: 10.3389/fmicb.2018.03220
195. White G. Oral health: A window into general health. *J Oral Biol* (2014) 1(1):5. doi: 10.13188/2377-987X.1000002

196. Oz HS, Chen T, Ebersole JL. A model for chronic mucosal inflammation in IBD and periodontitis. *Dig Dis Sci* (2010) 55(8):2194–202. doi: 10.1007/s10620-009-1031-x
197. Pietropaoli D, Del Pinto R, Corridoni D, Rodriguez-Palacios A, Di Stefano G, Monaco A, et al. Occurrence of spontaneous periodontal disease in the SAMP1/YitFc murine model of Crohn disease. *J Periodontol* (2014) 85 (12):1799–805. doi: 10.1902/jop.2014.140316
198. Vila AV, Imhann F, Collij V, Jankipersadsing SA, Gurry T, Mujagic Z, et al. Gut microbiota composition and functional changes in inflammatory bowel disease and irritable bowel syndrome. *Sci Trans Med* (2018) 10(472): eaap8914. doi: 10.1126/scitranslmed.aap8914
199. Lloyd-Price J, Arze C, Ananthakrishnan AN, Schirmer M, Avila-Pacheco J, Poon TW, et al. Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* (2019) 569(7758):655–62. doi: 10.1038/s41586-019-1237-9
200. Sberro H, Fremin BJ, Zlitni S, Edfors F, Greenfield N, Snyder MP, et al. Large-scale analyses of human microbiomes reveal thousands of small, novel genes. *Cell* (2019) 178(5):1245–59.e1214. doi: 10.1016/j.cell.2019.07.016
201. Carr VR, Witherden EA, Lee S, Shoaie S, Mullany P, Proctor GB, et al. Abundance and diversity of resistomes differ between healthy human oral cavities and gut. *Nat Commun* (2020) 11(1):1–10. doi: 10.1038/s41467-020-14422-w
202. Proctor LM, Creasy HH, Fettweis JM, Lloyd-Price J, Mahurkar A, Zhou W, et al. The integrative human microbiome project. *Nature* (2019) 569 (7758):641–8. doi: 10.1038/s41586-019-1238-8
203. Sasaki N, Miyamoto K, Maslowski KM, Ohno H, Kanai T, Sato T. Development of a scalable co-culture system for gut anaerobes and human colon epithelium. *Gastroenterology* (2020) 159(1):388–90.e5. doi: 10.1053/j.gastro.2020.03.021
204. Irving J, Socransky S, Heeley J. Histological changes in experimental periodontal disease in gnotobiotic rats and conventional hamsters. *J Periodontal Res* (1974) 9(2):73–80. doi: 10.1111/j.1600-0765.1974.tb00656.x
205. Agossa K, Dendooven A, Dubuquoy L, Gower-Rousseau C, Delcourt-Debruyne E, Capron M. Periodontal manifestations of inflammatory bowel disease: emerging epidemiologic and biologic evidence. *J Periodontal Res* (2017) 52(3):313–24. doi: 10.1111/jre.12422
206. Marchesan J, Girnary MS, Jing L, Miao MZ, Zhang S, Sun L, et al. An experimental murine model to study periodontitis. *Nat Protoc* (2018) 13 (10):2247–67. doi: 10.1038/s41596-018-0035-4
207. Ebersole J, Kirakodu S, Chen J, Nagarajan R, Gonzalez O. Oral microbiome and gingival transcriptome profiles of ligature-induced periodontitis. *J Dental Res* (2020) 99(6):746–57. doi: 10.1177/0022034520906138
208. Spekhorst LM, Imhann F, Festen EA, Van Bodegraven AA, de Boer NK, Bouma G, et al. Cohort profile: design and first results of the Dutch IBD Biobank: a prospective, nationwide biobank of patients with inflammatory bowel disease. *BMJ Open* (2017) 7(11):e016695. doi: 10.1136/bmjopen-2017-016695
209. Smillie CS, Biton M, Ordovas-Montanes J, Sullivan KM, Burgin G, Graham DB, et al. Intra-and inter-cellular rewiring of the human colon during ulcerative colitis. *Cell* (2019) 178(3):714–30.e722. doi: 10.1016/j.cell.2019.06.029
210. Tang Z-Z, Chen G, Hong Q, Huang S, Smith HM, Shah RD, et al. Multi-omic analysis of the microbiome and metabolome in healthy subjects reveals microbiome-dependent relationships between diet and metabolites. *Front Genet* (2019) 10:454. doi: 10.3389/fgene.2019.00454
211. Falvey JD, Hoskin T, Meijer B, Ashcroft A, Walmsley R, Day AS, et al. Disease activity assessment in IBD: clinical indices and biomarkers fail to predict endoscopic remission. *Inflamm Bowel Dis* (2015) 21(4):824–31. doi: 10.1097/mib.0000000000000341
212. Kugathasan S, Denson LA, Walters TD, Kim M-O, Marigorta UM, Schirmer M, et al. Prediction of complicated disease course for children newly diagnosed with Crohn's disease: a multicentre inception cohort study. *Lancet* (2017) 389(10080):1710–8.
213. Martin JC, Chang C, Boschetti G, Ungaro R, Giri M, Grout JA, et al. Single-cell analysis of Crohn's disease lesions identifies a pathogenic cellular module associated with resistance to anti-TNF therapy. *Cell* (2019) 178(6):1493–508.e1420. doi: 10.1016/j.cell.2019.08.008
214. Chen Y, Chen X, Yu H, Zhou H, Xu S. Oral Microbiota as Promising Diagnostic Biomarkers for Gastrointestinal Cancer: A Systematic Review. *OncoTargets Ther* (2019) 12:11131. doi: 10.2147/OTT.S230262
215. Liebsch C, Pitchika V, Pink C, Samietz S, Kastenmüller G, Artati A, et al. The saliva metabolome in association to oral health status. *J Dental Res* (2019) 98 (6):642–51. doi: 10.1177/0022034519842853

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 Impact of Oral-Gut Inflammation in Cerebral Palsy

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Background: Oral-gut inflammation has an impact on overall health, placing subjects at risk to acquire chronic conditions and infections. Due to neuromotor disturbances, and medication intake, cerebral palsy (CP) subjects present intestinal constipation, impacting their quality of life (QOL). We aimed to investigate how oral inflammatory levels predicted gut phenotypes and response to therapy.

Methods: A total of 93 subjects aging from 5 to 17 years were included in the study, and assigned into one of the 4 groups: CP with constipation (G1, $n = 30$), CP without constipation (G2, $n = 33$), and controls without CP with constipation (G3, $n = 07$) and without CP and without constipation (G4, $n = 23$). In addition to characterizing subjects' clinical demographics, medication intake, disease severity levels, salivary cytokine levels [TNF-α, interleukin (IL)-1β, IL-6, IL-8, IL-10], and Caregiver Priorities and Child Health Index of Life with Disabilities (CPCHILD). Statistical significance was evaluated by Shapiro-Wilks, Student's *T*-Test, ANOVA, and ANCOVA analysis.

Results: Salivary proinflammatory cytokines were highly correlated with the severe form of gut constipation in G1 ($P < 0.001$), and out of all cytokines IL-1β levels demonstrated highest correlation with all gut constipation ($P < 0.05$). A significant relationship was found between the type of medication, in which subjects taking Gamma-Aminobutyric Acid (GABA) and GABA+ (GABA in association with other medication) were more likely to be constipated than the other groups ($P < 0.01$). Cleary salivary inflammatory levels and gut constipation were correlated, and impacted QOL of CP subjects. G1 presented a lower QOL mean score of CPCHILD (49.0 ± 13.1) compared to G2 (71.5 ± 16.7), when compared to G3 (88.9 ± 7.5), and G4 (95.5 ± 5.0) ($P < 0.01$). We accounted for gingival bleeding as a cofounder of oral inflammation, and here were no differences among groups regarding gender ($P = 0.332$) and age ($P = 0.292$).

Conclusions: Collectively, the results suggest that saliva inflammatory levels were linked to gut constipation, and that the clinical impact of medications that controlled gut was reliably monitored via oral cytokine levels, providing reliable and non-invasive information in precision diagnostics.

Keywords: cerebral palsy, inflammation, cytokines, quality of life, caregiver priorities and child health index of life with disabilities, constipation, antiepileptic drug

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INTRODUCTION

Cerebral palsy (CP) is a life-limiting and costly disability characterized by a permanent neuromotor disorder affecting movement and by non-progressive degeneration of the brain (1, 2). As the major etiological factor for severe disability, the estimated prevalence of CP ranges from 2.3 to 2.9 per 1,000 live births observed in the United States (2011–2012 National Survey of Children's Health and the 2011–2013 National Health Interview Survey) (3). Children with CP experience limitations to everyday activities, adversely influencing their quality of life (QOL). CP can be accompanied by difficulties related to perception, sensation, behavior, cognition, communication, as well as epilepsy. Furthermore, CP subjects' QOL can be impacted by the development and progression of musculoskeletal disturbances such as oral-gut motor impairment through muscle spasticity (1).

Due to an abnormal increase in muscle tone and injury to neural pathways, spasticity acts as a negative factor in the lives of 85–90% of subjects with CP (1, 4, 5), impacting understanding subjects overall quality of life. The progression and severity of movement disability is classified by the Gross Motor Function Classification System (GMFCS) (6). GMFCS is stratified into five levels of body mobility: Level I (walking without limitations), Level II (walking with limitations), Level III (walking using a hand-held mobility device), Level IV (self-mobility with limitations), and Level V (transported in a wheelchair) (6). Our previous studies demonstrated that out of 254 subjects, 50 (19.7%) had GMFCS I, II, or III while 204 (80.3%) presented GMFCS IV or V (7–9). Notably, epilepsy prevalence increases in spastic CP subjects at the highest GMFCS classifications (IV and V) (10). Thus, there is an imminent need to discover non-invasive and precise diagnostics to predict these phenotypes seen in CP.

Gut constipation and lack of gastrointestinal control is highly prevalent in CP subjects, varying from 25 to 74% (3). Constipation is caused by multiple factors including: (1) reduced intake of fiber and liquids (responsible for the digestive system functioning) (11), (2) central nervous system damage (12), (3) mobility reduction (13), (4) and/or the use of antiepileptic drugs (AEDs) (11, 14). In subjects using AEDs the incidence of gut constipation is elevated (7). Other side effects co-occurring with gastrointestinal complications include oral dysbiosis, gingival bleeding (GB), and an increase of systemic inflammation (15). GB is found at a high frequency that may be due to the same factors that predispose to tooth decay and lead to the accumulation of biofilm (16). Difficulties in performing daily oral hygiene, intraoral sensitivity and orofacial motor dysfunction are the main contributing factors (17). Because epilepsy affects 77% of the subjects with CP, and standard clinical treatment for epileptic CP subjects is based on therapy with antiepileptic drugs (AEDs) (18). As the principal inhibitory neurotransmitter of the central nervous system and also a regulatory signal for muscle tone, Gamma-Aminobutyric Acid (GABA) is used for neuronal excitability control (e.g.,: benzodiazepines, phenobarbital, topiramate, vigabatrin, gabapentin enacarbil, and ezogabine). Side effects of AEDs can range from subjective reports of mild drowsiness to life-threatening neurologic and gastrointestinal consequences (19).

In addition to motor disturbances, CP subjects present impairment in inflammatory pathways. Epidemiological studies on premature births correlate the presence of high inflammatory levels in the umbilical cord, amniotic fluid, and fetal blood with white matter injury. In fact, premature babies are born in a state of severe inflammation (20–22). While CP and developmental impairment have diverse etiology, at the center of disease development, impaired inflammation regulates CP clinical phenotypes. Recently, attributed to dysregulated cytokine production, CP inflammation is modulated mainly by diet restrictions, gut dysfunction, medication intake. In epileptic subjects, seizures alone are shown to stimulate the synthesis of pro-inflammatory and pro-convulsive cytokines (23). IL-1β is a mediator of inflammatory response, cell death by apoptosis and regulator of bone resorption. This is a central cytokine modulated by master gene, such as inflammasome regulates how the host response is shaped in chronic conditions, including gut and oral dysbiosis, such as inflammatory bowel disease and periodontitis (24). In addition, other factors such as TNF-α correlate have shown synergistic actions with IL-1β, especially when in exacerbation response (25). While IL-6 levels have also been found in the crevicular gingival fluid in disease subjects (26), a chemokine interleukin-8 (IL-8 or CXCL8) is key migrating agent for myeloid-derived cells, such as neutrophils (27). In contrast, to potent activators of inflammation, several molecules counteract their actions. Central to IL-1β, feedback look is the production of interleukin-10 (IL-10) in a timely manner to spatially regulate tissues to return to homeostasis by potent actions (28). Inflammatory levels are the target of direct actions of drugs, and as side effects. AEDs can influence the immune system by modifying interleukin and chemokine concentrations in blood; these changes seem to be independent of the serum concentrations of these drugs (29, 30). In CP investigations, cytokine levels have been less investigated, especially in the context to their comprehensive clinical phenotypes, and in the context of response to essential therapeutics, which is important to their survival.

Here we investigated how oral inflammatory signatures correlated with gut constipation and impacted the QOL of each subjects. Our findings indicated significant correlations between proinflammatory cytokine compositional changes detected in saliva of CP patients and gut constipation, and medication intake. The specific correlation of severe forms of gut constipation with IL-1β cytokine was specific and did not include other inflammatory factors significantly, was confirmed by cofounder evaluations. While we did not include dental plaque, we accounted for gingival bleeding as a cofounder of oral

Abbreviations: CP, Cerebral Palsy; GABA, Gamma-Aminobutyric Acid; GABA+, Gamma-Aminobutyric Acid in association with other medication; CPCHILD, Caregiver Priorities and Child Health Index of Life with Disabilities; QOL, Quality of life; GMFCS, Gross Motor Function Classification System; AED, Antiepileptic drug; AEDs: Antiepileptic drugs; GB, Gingival Bleeding; GI, Gingival Index; BSS, Bristol Stool Scale; CPQoL-Child, Cerebral Palsy Quality of Life Questionnaire for Children Child Report; IL-1β, Interleukin-1 beta; IL-6, Interleukin-6; IL-8, Interleukin-8; CXCL8, Chemokine C-X-C motif ligand 8; IL-10, Interleukin-10; TNF-α, Tumor necrosis factor alpha.

inflammation, and there were no differences among groups regarding gender, and age. In addition, we assayed all clinical factors that impacted the patient's QOL and each cytokine level. Clearly, medication intake was a clinical factor with direct impact to gut constipation and consequently QOL. While our investigation cannot determine causation of clinical phenomic factors that were impacted by AEDs, including dental plaque or medication biochemical actions, positive correlation by host salivary inflammatory levels was specific for certain types of cytokines and not all the molecules assays, demonstrating the direction of future research. We propose that this data reveals a much greater importance of salivary cytokines for evaluation of systemic health and their gut response to various clinical challenges. Here, we aimed to investigate how salivary inflammatory levels predicted intestinal phenotypes. Because inflammation impacts the overall subject, we further investigated how drug intake and clinical factors control overall QOL of subjects with CP. Altogether, the results suggest that saliva inflammatory levels were linked to gut constipation, an innovative and non-invasive method to monitor QOL, improving precision medicine and dentistry.

MATERIALS AND METHODS

Study Design

This study was reviewed and approved by the Research Ethics Committee of the Cruzeiro do Sul University-Brazil Platform, São Paulo, Brazil (IRB #2,452,626). Written informed consent was obtained from the guardian of each child or adolescent after they were informed about the study. A cross-sectional study was performed with subjects with spastic CP diagnosis, who received a physical rehabilitation treatment at a referral center in São Paulo, Brazil, at the time of data collection.

Participants

Seventy subjects with a medical diagnosis of CP were invited to participate in this study at Disabled Child Care Association (AACD) in São Paulo, Brazil. Inclusion criteria were a medical diagnosis of spastic CP ranging from 5 to 17 years, both male and female, and the presence or absence of constipation. Subjects who presented progressive or neurodegenerative lesions or uncooperative behavior were excluded during clinical oral examinations. Seven subjects with CP were excluded from the research because they did not collaborate during the oral exams. The control group consisted of 30 normotypic subjects who attended the Pediatric Dentistry Clinic of the Faculty of Dentistry of the Cruzeiro do Sul University, residing in the same health district as the study group (that is, with the same health practices developed in health services), at the time of data collection. The final sample of the study was composed of subjects assigned into one of the 4 groups (G1-4) based on the prevalence of (1) CP and (2) Constipation. Subjects were assigned as CP with constipation (G1, $n = 30$), CP without constipation (G2, $n = 33$), and controls without CP with constipation (G3, $n = 07$) and without CP and without constipation (G4, $n = 23$). Both demographic and clinical data were collected for each subject from the year 2018 until 2019. Data regarding gender, age, race (white, black and others), caregiver occupation and education, family income, the medical diagnosis of CP according to the type of movement disorder (spastic), clinical pattern: tetraplegia (a system-wide decay of neuromotor function, represented by increased muscle tone in all four limbs and trunk involvement); diplegia (increased muscle tone in lower limbs, but may affect upper limbs but to a lesser extent), and hemiplegia (or increased muscle tone in a hemibody), furthermore GMFCS (levels I–V) (6) and use of AEDs were collected from their medical records. Caregivers answered the Caregiver Priorities and Child Health Index of Life with Disabilities (CPCHILD) (31) that consists of 36 items rated in six sections that are scaled from 0 (worst) to 100 (best) and were averaged to determine overall QOL. CPCHILD is represented by the following domains: (1) Personal Care (eight items); (2) Positioning, Transfer, and Mobility (eight items); (3) Communication and Social Interaction (seven items); (4) Comfort, Emotions, and Behavior (nine items); (5) Health (three items); and (6) Overall Quality Of Life (one item).

Gastrointestinal Constipation

This study adopted the clinical constipation definition proposed by the Bristol Stool Scale (BSS) for constipation (32). The translation and adaptation of the BSS into Brazilian Portuguese showed high reliability, indicating its usefulness in the practical clinic for the purpose for which it was planned, both for use in children (33) and adults (34).

Saliva Collection

Unstimulated whole saliva samples were collected in dental assessment sessions. Subjects were asked to refrain from eating, drinking liquids, or brushing their teeth for at least 1 h prior to saliva collection. The collection was performed with the subjects sitting comfortably in a bright and ventilated room. Whole saliva was collected by passive flow for 5 min. After collection, the Salivette® was centrifuged at 5,000 rpm for 5 min at 4°C (Hettich Centrifuge, model Universal 320R, Tuttlingen, Germany) and frozen in a freezer at −80°C.

Biomarker Sub-analysis

For a subset of 37 participants, all of whom were CP subjects, we analyzed the salivary cytokines IL-1β, IL-6, IL-8, IL-10, and TNF-α. The analysis of cytokines in saliva was performed using a CBA Cytokine Inflammatory Kit (Becton Dickinson, CA, USA) for the detection of TNF-α, IL-1β, IL-6, IL-8, IL-10. All analyses were performed in duplicate. Briefly, 25μL of fluorescent particles conjugated to antibodies specific for each cytokine were added to 25 μL of the saliva and incubated for 1 h at room temperature away from light. Subsequently, 25 μL of the secondary antibody conjugated to a fluorochrome was added to the mixture and incubated for 2 h at room temperature. The results were compared to a standard curve with serially diluted cytokines. The particles were washed to remove unbound antibodies, resuspended in the wash buffer, and analyzed using a BD Accuri (BD Biosciences). Data acquisition was performed using BD-Accuri C6 Software, and concentrations were determined using FCAP software v.3.0 (BD Biosciences).

Gingival Index

The evaluation of oral dysbiosis was assayed through the GI (35) by using a millimeter plastic periodontal probe (HuFriedy's Colorvue PerioScreen Kit probe, Chicago, IL, USA), which was gently passed in the gingival margin of all teeth, in reference to the distobuccal papilla, the buccal margin, the mesiobuccal papilla, and the lingual/palatine margin. Partially erupted teeth and residual roots were excluded without replacement. The index was calculated by the percentage of the sum of the subjects values of each tooth divided by the number of faces examined. Classified as positive for gingivitis were the subjects that presented gingival marginal bleeding more than 10% of the total sites evaluated (36). Gingival index was included as a surrogate for oral hygiene.

Statistical Analyses

Analyses of descriptive statistics were performed to characterize the sample, calculate measures of central tendency and variability for the quantitative variables. The normality assumption of the quantitative variables was evaluated using the Shapiro-Wilks test. When normal distribution was observed, parametric tests were performed. Otherwise, non-parametric tests were selected to determine the significance of intergroups differences. Student's *T*-Test was used for any comparison of two groups (including intergender groups and comparisons of constipated and non-constipated subjects), while two-tailed ANOVA (comparison without covariance correction) and ANCOVA (comparison with covariance correction) were used in comparisons of experimental group, age, cytokine measures, and gingival index. IBM SPSS Statistics (SPSS for Windows, Version 20.0, Armonk, NY: IBM Corp.) and RStudio were used for analyses, with significance thresholds at $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$. Data was visualized using RStudio with the ggplot2 package and assisted by the dplyr and tidyr packages.

RESULTS

Demographics

The sample power was calculated using means and standard deviations of overall domains among G1 (51.0 ± 13.1), G2 (28.5 ± 16.7), G3 (11.1 ± 7.5) and G4 (4.5 ± 5.0) (37). The results showed that the G*Power at the 95% confidence interval was 96.88%.

To predict salivary cytokine levels with gut phenotype, we first investigated how overall clinical factors impacted both oral and gut phenotypes in CP subjects. We collected subjects detailed information including demographics, clinical factors, and AEDs as potential influencers of a subject's inflammation, wellbeing, and QOL (**Table 1**). In our population, 47 of our 93 subjects were female and 46 were male, accurately representing the relatively equal ratio of female to male.

G1 was composed of 30 non-ambulatory subjects (GMFCS levels IV and V) and G2 of 14 ambulatory subjects (GMFCS levels I, II, and III) ($P < 0.0001$). Lower QOL was observed for constipated subjects presenting the clinical pattern tetraplegia when compared to non-constipated CP ($P < 0.001$) (**Table 1**). Interestingly, gender was significantly associated with constipation ($P < 0.05$) with females appearing more likely to be constipated in our population. There were no significant differences among groups regarding gender ($P = 0.332$), age ($P = 0.291$), race ($P = 0.08$), and education ($P = 0.5628$); however, significant differences were found on the basis of caregiver occupation ($P < 0.01$) presenting the caregivers CP group a higher number of unemployed, and lower family income ($P < 0.05$).

Distribution of age in our population shows a peak at approximately 6 years of age, with a slight steady dropoff in population representation until approximately age 14, with a steep dropoff in population representation from ages 14–19 (**Figure 1A**). It is important to note once more that the GMFCS levels are dependent on CP diagnosis, as tetraplegia, diplegia, and hemiplegia insinuate a certain level of mobility reduction (Pearson $R = 0.787$). We found this correlation to be pertinent in further analyses of GB, salivary cytokines, and QOL.

CP Diagnosis

When diagnosing CP, there are three widely accepted presentations: (1) hemiplegic; (2) diplegic; and (3) tetraplegic. In our population, the distribution of subjects is made up of these three groups as well as healthy subjects (control). In our population 9.68% of subjects were hemiplegic, 26.88% diplegic, 31.18% tetraplegic, and 32.26% normotypic (**Figure 1B**). CP diagnosis was found to be significant in our population ($P < 0.001$) in determining QOL (**Table 1**). Another datapoint we used to determine the severity of a patient's condition was motor function ability as classified by the GMFCS scale, which rates a child's movement from I (walking without limitations) to V (total motor dependence). The GMFCS on our population, levels IV (29.03%) and V (34.92%) make up the majority of our population and are reserved for subjects with clinically advanced CP, namely diplegic and tetraplegic CP (**Figure 1C**). We found that a subject's GMFCS level was statistically significant in determining their QOL ($P < 0.001$). This significance reflects the impact the severity of cortex injury on functional motor independence; diplegic and quadriplegic subjects are limited exclusively to GMFCS IV and V and have a significant motor impairment that requires powered mobility assistance and/or a physically able caretaker.

Constipation

Indirect symptoms of CP are not limited to the oral cavity: of our 63 subjects who presented CP, 30 were constipated at the time of examination, representing a much higher prevalence of constipation (47.62%) compared to the estimated prevalence of 16% in the global population (38).

According to CPCHILD, the groups differed significantly ($P < 0.05$). G1 presented lower values for all sections of the questionnaire when compared to G2, G3, and G4. In our cohort, we found constipated subjects to have significantly ($P < 0.0001$) lower QOL (56.09 ± 21.86) than those who were non-constipated (82.40 ± 19.35). In **Figure 2**, this difference is visualized, showing continuity in this hypothesis when stratified by gender. While future studies are required to investigate the full extent of this association, it can be concluded that constipated subjects in our cohort were statistically more likely to present a

TABLE 1 | Population-wide demographics table.

	G1 - constipated CP[a]	G2-No constipated CP[a]	G3- constipated control[a]	G4- no constipated control[a]	*p*-value*
DEMOGRAPHICS					
Total Subjects	30 (32.25%)	33 (35.48%)	7 (7.52%)	23 (24.73%)	
Gender					
Female	19 (66.3%)	14 (42.4%)	4 (57.1%)	10 (43.5%)	0.332
Male	11 (36.7%)	19 (57.6%)	3 (42.9%)	13 (56.5%)	
Age (mean±SD)	8.5 ± 4.1	10.3 ± 3.7	8.8 ± 4.5	10.2 ± 4.3	0.291
Race					
White	19 (63.4%)	15 (45.5%)	6 (85.7%)	18 (78.3%)	
Others	9 (30%)	16 (48.5%)	0 (0%)	5 (21.7%)	0.08
Black	2 (6.4%)	2 (6%)	1 (14.3%)	0 (0%)	
Occupation (Caregiver)					
Employed	11 (36.66%)	13 (39.39%)	5 (71.42%)	19 (82.60)	0.0017*
Education					
≤8 years	21 (70%)	23 (69.69%)	4 (57.15%)	14 (60.86%)	0.5628
>8 years	9 (30%)	10 (30.31%)	3 (42.85%)	9 (39.14%)	
Family (income)					
≥3MW	4 (13.34%)	9 (27.28%)	3 (42.86%)	11 (47.82%)	0.018*
<3MW	26 (86.66%)	24 (72.72%)	4 (57.14%)	12 (52.17%)	
ORAL HEALTH- PERIODONTAL PARAMETERS					
Gingival bleeding	32.4 ± 19.8	4.1 ± 3.5	0.6 ±1.4	0.1 ± 0.6	<0.001*
Gingivitis					
Presence	23 (76.7%)	2 (6.1%)	0 (0%)	0 (0%)	<0.001*
Absence	7 (23.3%)	31 (93.9%)	7 (100%)	23 (100%)	
SYSTEMIC HEALTH- CEREBRAL PALSY					
Clinical pattern					
Tetraplegia	20 (66.66%)	9 (27.27%)	0 (0%)	0 (0%)	0.0009*
Diplegia	10 (33.33%)	15 (45.45%)	0 (0%)	0 (0%)	
Hemiplegia	0 (0%)	9 (27.27%)	0 (0%)	0 (0%)	
GMFCS					
I - II – III	0 (0%)	14 (42.4%)	not applicable	not applicable	<0.0001*
IV – V	30 (100%)	19 (57.6%)	not applicable	not applicable	
Medication					
GABA	5 (16.7%)	1 (3%)	0 (0%)	0 (0%)	
GABA+	9 (30%)	2 (6.1%)	0 (0%)	0 (0%)	
Sodium	5 (16.7%)	4 (12.1%)	0 (0%)	0 (0%)	0.0063*
Calcium	1 (3.3%)	1 (3%)	0 (0%)	0 (0%)	
No medication	10 (33.3%)	25 (75.8)	7 (100%)	23 (100%)	
Quality of life					
Personal care	20.2 ± 21.6A	53.6 ± 37.5B	80.0 ±23.8C	96.2 ± 8.4C	<0.05*
Positioning, transfer and mobility	29.3 ± 26.1A	57.9 ± 34.4B	100.0 ±0.0C	99.9 ± 0.5C	<0.05*
Communication and social interaction	55.6 ± 28.3A	82.1 ± 19.3B	100.0 ±0.0C	100.0 ±0.0C	<0.05*
Comfort, emotions and behavior	55.6 ± 22.7A	81.2 ± 16.00B	91.3 ±12.8C	96.1 ± 4.9C	<0.05*
Health	73.6 ± 18.2A	83.8 ± 12.9B	90.3±10.2C	96.5 ± 5.7C	<0.05*
Overall quality of life	55.7 ± 24.3A	70.3± 15.2B	71.4 ±19.5C	81.7 ± 20.8C	<0.05*
Domains means	49.0 ± 13.1A	71.5 ±16.7B	88.9 ±7.5C	95.5 ±5.0C	<0.05*

*[a]Values represent mean ± SD or percentage (%); *p-values for group comparisons were significant at 0.05; Chi-square, ANOVA, One-Way, Kruskal-Wallis tests. Different letters denote statistically significant differences (p < 0.05). Uppercase letters compare values horizontally (intragroup evaluation). SD, standard deviation; BOP, Bleeding on probing; MW, minimum wage, unit for measuring income in Brazil, broadly corresponding to $251 U.S. during the study period.*

Numbers and relevant statistics for study groups. Groups are defined as G1 (CP with constipation), G2 (CP without constipation), G3 (control with constipation), and G4 (control without gut constipation).

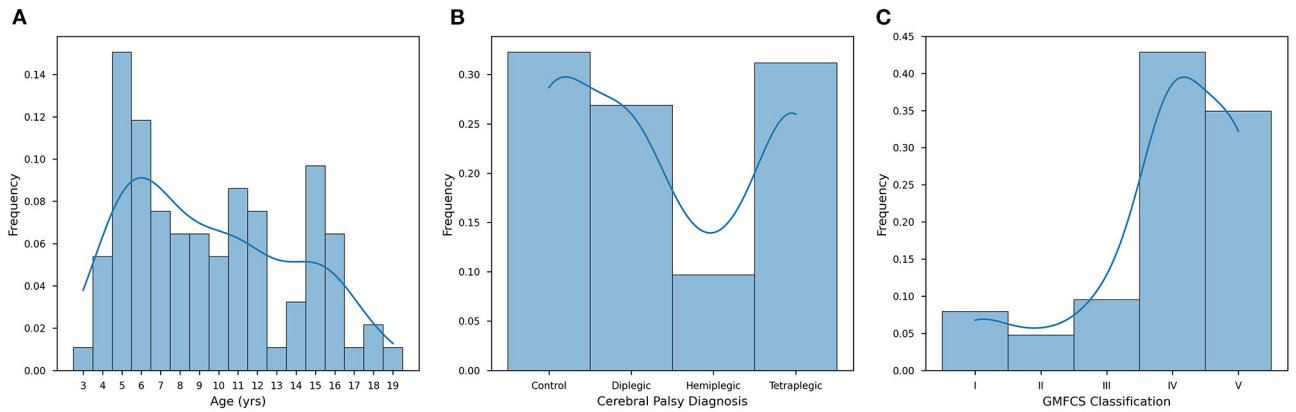

FIGURE 1 | Distribution patterns of the CP study population. **(A)** Individuals from the cohort aged from 3 to 19, with a mean age of 9.57 ± 4.20 years ($n = 93$). **(B)** The study population was composed of healthy individuals (controls, 32.26%), tetraplegics (32.26%), diplegics (26.88%), and hemiplegics (9.68%). **(C)** Distribution is measured along the GMFCS scale from I-V for only G1 and G2. Stages I-III are considered ambulatory, while stages IV and V require significant assistance (typically a wheelchair or mobility scooter). Stages I, II, and III are less represented (7.94%, 4.76%, and 9.52%, respectively), while stages IV (42.86%) and V (34.92%) make up the vast majority of G1 and G2 ($n = 93$). For each of these histograms, an LM regression is overlaid to make visual trend observation more convenient.

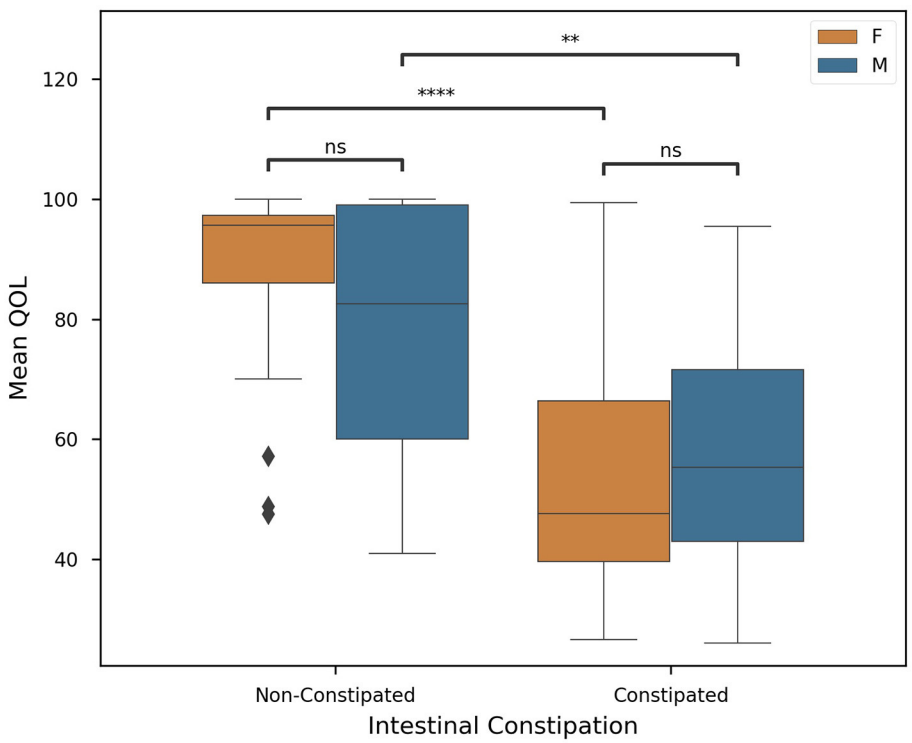

FIGURE 2 | Quality of life in individuals with and without constipation according to sex. G1 presented lower values for all sections of the questionnaire when compared to G2, G3, and G4. In our cohort, we found constipated subjects to have significantly ($p < 0.0001$) lower QOL (56.09 ± 21.86) than those who were non-constipated (82.40 ± 19.35) ($n = 93$, significance was evaluated by two-tailed ANOVA, p-value < 0.05). Welch's t-tests were employed with Bonferroni corrections for (1) intersex intragroup comparisons and (2) intergroup intrasex comparisons (Key: ns - $p \geq 0.05$, **$p < 0.01$, ****$p < 0.0001$).

lower mean quality of life. In **Supplementary Figures 1A–E**, we plotted each subscore against overall QOL and we observed that higher intensity of discomfort hurt the physical and psychological quality of life.

The primary factor affecting constipation and QOL in our population was revealed to be the type of medication a patient was prescribed. Here we found that the choice of AED used to combat spasticity was significantly correlated with the QOL (*P*

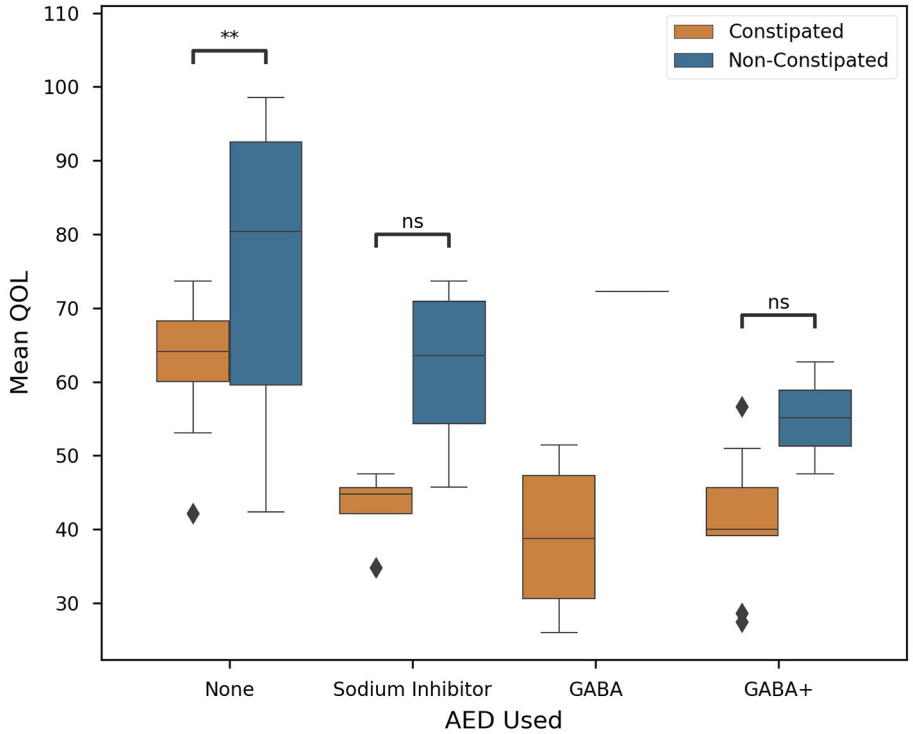

FIGURE 3 | Correlation of clinical factors in CP patients. Constipated subjects are in orange, non-constipated in blue. Colored boxes show interquartile range, black line marks the median, and whiskers show the largest outliers within 150% the interquartile range above the 75th percentile and below the 25th percentile. Remaining outliers are marked with points. Standard box plot comparing AED used and Quality of Life. AED used is significantly correlated with QOL ($P < 0.0001$), $n = 63$, and significance was evaluated by two-tailed ANOVA, p-value < 0.05. Where possible (>1 subject), Welch's t-tests were employed for each medication on the basis of constipation (Key: ns - $p \geq 0.05$, **$p < 0.01$).

< 0.0001) of our cohort. In **Figure 3**, AED use is plotted against QOL. We noticed that, in particular, GABA and GABA+ subjects presented lower mean QOL (43.89 ± 12.61) than other groups (78.20 ± 21.39). Meanwhile, sodium inhibitors presented a much higher mean QOL (51.26 ± 12.29). This finding is exceedingly promising, but future investigation is necessary to further explore sodium inhibitors and their effect on QOL.

However, we found that while both AED prescription and intestinal constipation are significant in determining a subject's QOL, ANCOVA revealed that the interaction between these two variables is insignificant ($P = 0.787$). Future studies are required to determine if QOL can be determined solely by the medication or gut health of a patient.

Salivary Cytokines

In many subjects, the lack of an ability to care for oneself leads to the development of oral and gum disease. If a large number of teeth have bleeding gingiva, there is a high chance of oral disease manifesting itself as gingival inflammation and periodontitis.

For a subset of our population, we collected salivary cytokine measures to get a deeper understanding of systemic inflammation and its relationship with constipation and medication. For all cytokines for which data were collected (IL-1β, IL-6, IL-8, IL-10, and TNF-α), mean levels were increased in constipated subjects (**Figure 4**). Of these cytokines, only IL-8, IL-10, and IL-1β levels were significantly different on the basis of intestinal constipation ($P < 0.05$).

Salivary Cytokines as a Predictor of Intestinal Constipation

We first quantified salivary cytokines to predict intestinal constipation yields promising statistical correlations (**Figure 4**). Of the cytokines measured, IL-1β, IL-8, and IL-10 measures were statistically significant ($P < 0.05$) in determining the presence of intestinal constipation. However, remaining cytokine levels were insignificant in classifying the presence of constipation. Therefore, there exists a specific but significant associative pathway between constipation and cytokine levels.

Establishing Oral-Gut Axis for Diagnosis

We found in our cohort that AEDs and intestinal constipation are significantly associated with both GB and one another ($P < 0.0001$). We noticed that, in particular, GABA and GABA+ subjects presented higher GB ($39.39 \pm 22.86\%$) than other groups ($5.84 \pm 9.32\%$) (**Supplementary Figure 2F**). In this case, co-measuring AED use and intestinal constipation would prove a more statistically fortified marker for GB than either in isolation.

In the case of gender, we found females to have a mean GB ($15.82 \pm 22.16\%$) almost double that of males ($8.05 \pm$

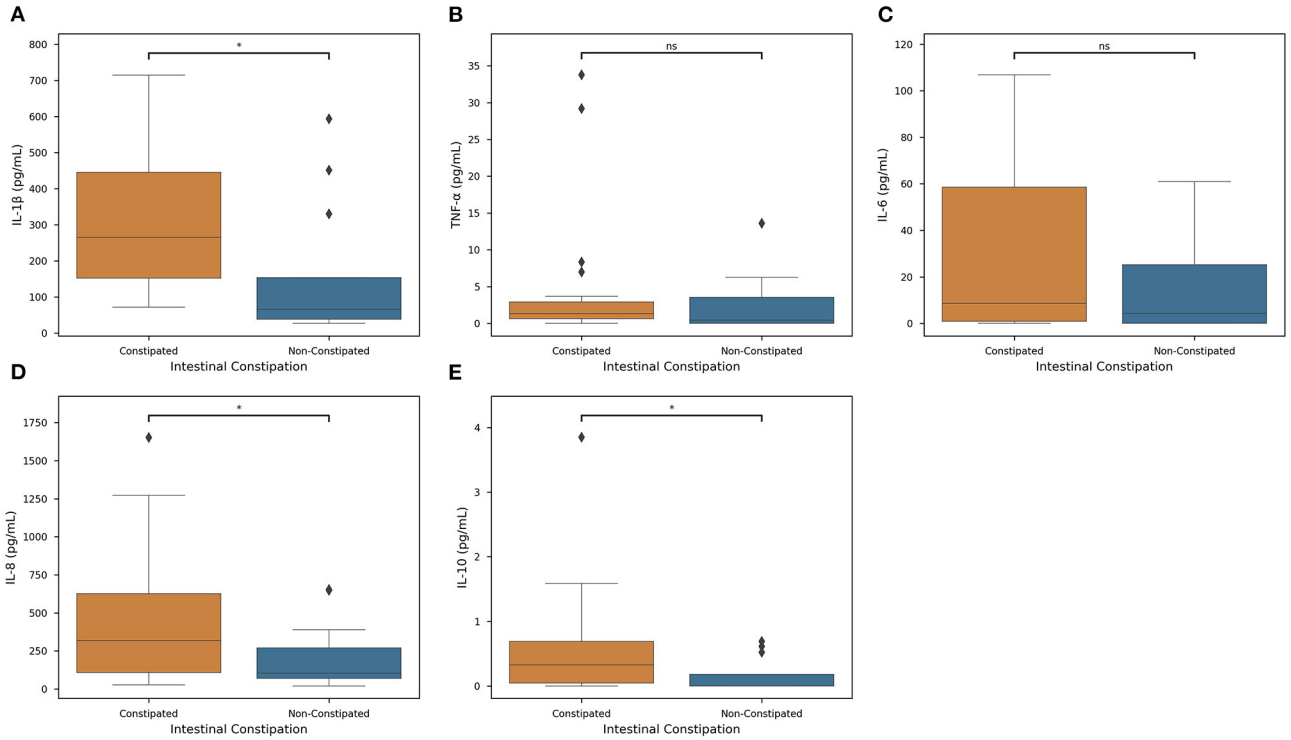

FIGURE 4 | Constipation actions on Inflammatory Cytokine Levels. Distributions of each measured cytokine—**(A)** IL-1β, **(B)** TNF-α, **(C)** IL-6, **(D)** IL-8, and **(E)** IL-10—in constipated subjects (orange) and non-constipated subjects (blue) were mapped. Colored boxes show interquartile range, black line marks the median, and whiskers show the largest outliers within 150% the interquartile range above the 75th percentile and below the 25th percentile ($n = 63$). For each boxplot, Welch's *t*-tests were employed to determine associations between each cytokine and constipation (Key: ns - $p \geq 0.05$, *$p < 0.05$).

12.04%, **Supplementary Figure 3A**). Overall mean GB was 12.08 ± 18.27%. In our population, we found gender ($P < 0.05$), CP diagnosis ($P < 0.0001$) and GMFCS classification ($P < 0.0001$) were found to be significantly correlated with GB, while age showed no such significance (**Supplementary Figure 3B**). Normotypic subjects (those who do not suffer from CP) have markedly lower mean GB 0.6% than any other group, with 2.79% mean hemiplegic GB, 12.19% mean diplegic GB, and 27.11% mean tetraplegic GB (**Supplementary Figure 3C**).

Our results demonstrated that IL-1β ($P < 0.0001$), IL-6 ($P < 0.05$), IL-8 ($P < 0.01$), and IL-10 were significantly associated with GB percentage. In **Supplementary Figure 2**, each cytokine was plotted against GB (%) in an effort to reveal trends that could be fortified by more rigorous statistical methods. In **Supplementary Figures 2A,C–E**, there is a clear trend indicating that GB was positively correlated (Pearson Correlation) with IL-1β ($R = 0.720$), IL-6 ($R = 0.343$), IL-8 ($R = 0.425$), and IL-10 ($R = 0.505$). In particular, it is important to consider IL-1β, IL-8, and IL-10 in our population, as their levels were significantly correlated with both systemic and oral clinical factors. Interestingly, we found in our population that QOL is more specific, as only IL-1β levels were significant in determining QOL ($P < 0.05$). Furthermore, the AED used by an individual was correlated with IL-1β ($P < 0.0001$) and IL-6 ($P < 0.05$) levels in our salivary cytokine subcohort (**Figure 5**). While remaining cytokines were not significantly correlated with AEDs, it is important to note that IL-1β was the sole cytokine correlated with QOL. In **Figure 6**, we plotted each cytokine against QOL. A visual trend connecting IL-1β levels to QOL (**Figure 6A**) corroborated our statistical findings. IL-10 levels were not significantly correlated with QOL ($P = 0.076$) showing in **Figure 6E**.

DISCUSSION

The results provided novel information related to the severity of CP, the presence of inflammation of these subjects, and the consequences of treatment on constipation, oral health, and quality of life. To the best of our knowledge, this is the first study to evaluate the association among salivary inflammation, intestinal constipation, and QOL of CP using the CPCHILD. According to the motor type disorder, subjects with CP are classified as at least one of spastic, dyskinetic (dystonic, choreic, and athetosic), or ataxic. The subjects presented spastic CP, the most prevalent type of motor disorder, with tetraplegia the clinical pattern most prevalent (32.26%) (**Figure 1B**). Consistent with our results, tetraplegia was also observed in 3,294 children studied in 18 states in the USA (32.9%) (39). Early brain damage triggers complex adaptive neuroplasticity processes involving multiple functional systems. Different factors interact with

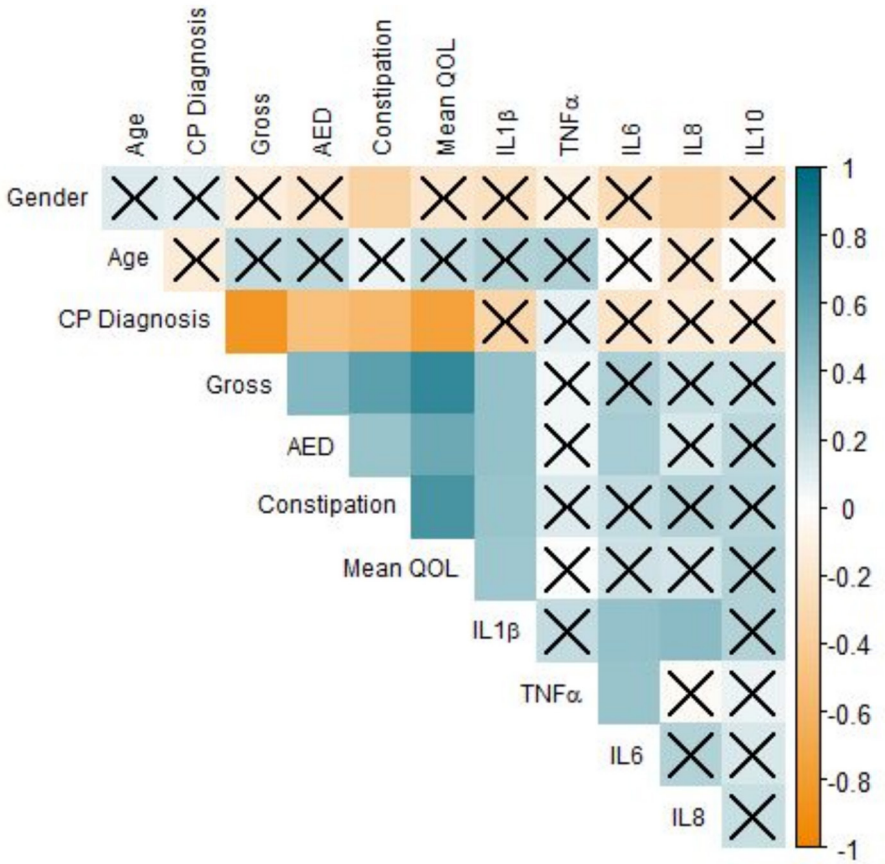

FIGURE 5 | Correlation of clinical factors in CP patients. Correlogram of Pearson's correlation of a subset of clinical factors influencing CP patients. Specificity of our dataset drove subset selection criteria: many factors were removed from this figure but included elsewhere to improve readability. Each axis is labeled by relevant factors to form a lower-triangle correlogram. Each box represents a correlation coefficient of two factors assigned, where size and saturation represents the magnitude of the correlation. Pearson score is represented from −1 (red) to +1 (blue), measuring the magnitude of correlation between the two variables. Boxes overlaid by an X represent associations that are not statistically significant ($P \geq 0.05$) regardless of the strength of correlation ($n = 63$, significance was evaluated by Pearson's correlation and student's *t*-test, p-value < 0.05).

brain plasticity potentials, influencing the natural history of cerebral palsy in the early years of childhood (40). The later the interdisciplinary intervention, the smaller the gain during brain plasticity, reducing the potential for improvement in the individual's condition.

Here, we observed a shortage of national instruments, which appears to reflect the difficulty of the scientific community to develop tools for assessing the quality of life that apply to the socio-cultural diversity of the country. For all questionnaires revised, only CPCHILD (31) and Cerebral Palsy Quality of Life Questionnaire for Children Child report (CPQol-Child) (41) are specific to CP subjects. CPQoL-Child is the individual's own report since it was mostly used in children with GMFCS level I or II and excluded the individual with any intellectual deficits and lack of an efficient communication system. In this study, the largest number of individuals belonged to GMFCS level IV and V (**Figure 1C**) and due to the difficulty of communication, their caregivers answered the questionnaire as their proxy. The Cronbach's alpha value estimated for the CPCHILD was 0.879. It was observed that G1 presented a lowest mean score of CPCHILD (51.0 ±13.1) compared to G2 (71.5 ± 16.7), G3 (88.9 ± 7.5), and G4 (95.5 ±5.0) ($P < 0.05$) (**Table 1**). Seventy percent of caregivers of CP subjects in this study had <8 years of study (**Table 1**). Intellectual capital, represented by education, increases the likelihood of access to information, health-related issues (42) facilitating understanding and recognizing the importance of self-care. A low level of caregiver education influences the child's overall health, represented by self-care of general and oral health (43).

The possible explanation for the lower education level of caregivers for individuals with CP is the "caregiver martyr syndrome" wherein a caregiver neglects their own health, studies, and finances to provide more frequent and consistent care (44). With a lower educational level and fewer paid employment opportunities, there is a direct logical connection linking significantly lower income to caregivers of individuals with CP (**Table 1**). Caregivers' potentially lower standard of education prevents them from entering the skilled labor market. Thus, they

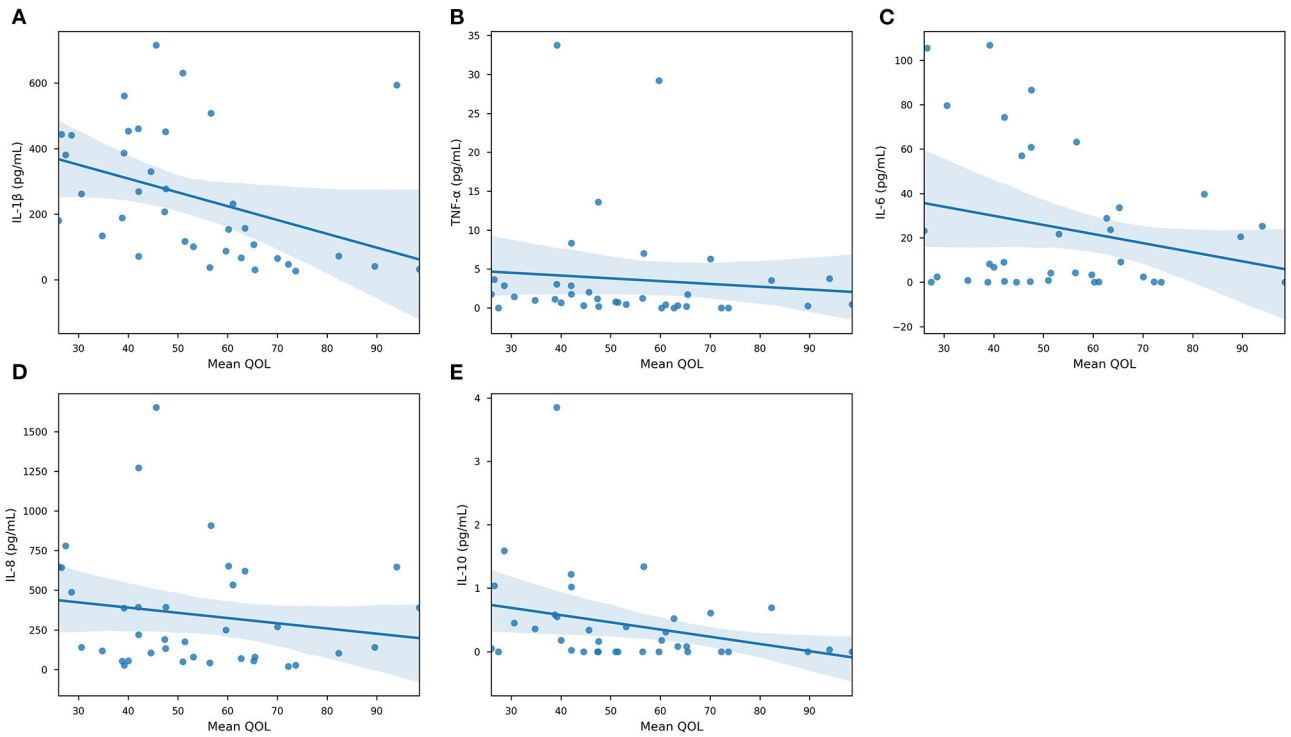

FIGURE 6 | Quality of Life association with specific cytokines. Scatterplots of collected cytokine levels —**(A)** IL-1β, **(B)** TNF-α, **(C)** IL-6, **(D)** IL-8, and **(E)** IL-10—and Quality of Life. Blue line marks the linearly regressed trendline (lm) and the shaded region corresponds to the 95% confidence interval. *P*-values from ANOVA are: IL-1β, $P < 0.05$—TNF-α, $P = 0.589$—IL-6, $P = 0.156$—IL-8, $P = 0.325$—IL-10, $P = 0.0762$ ($n = 63$, significance was evaluated by ANCOVA, p-value < 0.05).

dedicate themselves exclusively to household chores and taking care of the subject with CP (45).

We noticed that great discomfort has been associated with increased motor impairment and other comorbidities, particularly constipation and spasticity (**Figure 2**). In subjects with minor GMFCS (I, II, and III), the pain was observed during voluntary movements, whereas in subjects with higher GMFCS (IV and V), the pain was directly related to movement performed by the caregiver or therapist (46). Higher intensity of pain and discomfort had a negative effect on the physical and psychological quality of life (**Supplementary Figures 1A–E**). Communication and social interactions in subjects with CP in GMFCS level V present lower favorably and show large variation (47) (**Supplementary Figure 1C**). Fairhurst et al. concluded in 2018 that increasing awareness of CP pain and comorbidities, particularly overall health and constipation, can help prevent and treat the disorder more effectively, increasing patients' overall quality of life.

Medication such as AEDs are frequently used for the control of epileptic seizures (48). These drugs can be administered in monotherapy or polytherapy, depending on the response to seizure control (49). Results demonstrated that subjects with a lower quality of life were positively correlated to those who use polytherapeutic GABA treatments (GABA+), while those who use monotherapy, especially sodium channel inhibitors, have a better quality of life when compared with GABA and GABA+ (**Figure 3**). AEDs have an effect on voltage-dependent modulation of sodium, calcium, and potassium channels, and the GABA is considered the primary inhibitory neurotransmitter not only in the central nervous system but also on enteric nerves (49). However, AEDs aimed to achieve seizure control are usually limited by toxicity and adverse effects that impair an individual's QOL (50) (**Figure 3**). The subjects taking GABA or GABA association presented a negative correlation with the quality of life and also present intestinal constipation not only for the inhibitory GABA action (51), but may also be related to dysbiosis.

The use of GABA antiepileptic medication and associations to control seizures reduced salivary flow, increased salivary viscosity and gingival bleeding (**Supplementary Figure 2F**). Thus, the use of medication causes not only constipation but also gingival bleeding and increases the cytokines levels (**Supplementary Figures 2A–E**) (51). G1 presented significantly higher percentages of subjects with gingivitis compared to all other groups ($P < 0.001$). Additionally, CP subjects using GABA or GABA+ who were constipated had higher levels of GB than those who did not ($P = 0.006$, **Table 1**).

Accumulation of biofilm acts as a risk factor in the development of the main oral diseases, such as dental caries and periodontal disease. Subjects diagnosed with CP are more susceptible to poor oral hygiene due to neurological impairment and the presence of primitive oral pathological

reflexes, facilitating the progression of periodontal disease (52, 53). While a limitation of the study was the lack of evaluation of the oral hygiene index, gingival bleeding index was used as a surrogate of oral inflammation.

Current literature indicates that intestinal disorders play a prominent role in inflammatory responses to neurological conditions (54). This line of evidence is fundamental to identifying the effects of dysbiosis on mucosal inflammation throughout the digestive tract. Significantly higher levels of IL-1β, IL-6, IL-8, and IL-10 were found in constipated subjects with GB from this study (**Figure 4**), indicating an ongoing inflammatory process and the progression of periodontal disease (55–59). Since the use of these medications cause CP subjects to present a reduced salivary flow rate, an increased value of salivary osmolality, dry mouth, and gingivitis, which is represented by higher levels of inflammatory cytokines in quadriplegics (15). Sodium inhibitors, however, have a less severe correlation with GB ($R^2 = 0.684$) (**Supplementary Figure 2F**) but have markedly lower effects on a patient's QOL and they are associated with lower levels of constipation, it can be assumed that sodium inhibitors carry less of a systemic reaction in our study population (**Figure 3**).

Constipation is common comorbidity described in CP subjects (11), and current literature agrees that constipation is the causal factor of gut dysbiosis (60, 61). Constipation prevalence observed in this study was higher (47.62%). Constipation and intestinal dysbiosis leads to increased mucosal permeability (leaky-gut) (61) of the gut-brain axis (62) increasing serum endotoxin concentration. These endotoxins activate the immune system and promote IL-1β production as observed in our study (**Figure 4A**) and in individuals with Autism Syndrome Disorder (63). To date, no research has been published that evaluated the effect of dysbiosis on the development of gingivitis in individuals with cerebral palsy.

In addition to GI and oral inflammation, we have observed gender to be an important variable in regard to inflammation. Females presented higher pro-inflammatory cytokine expression when compared to males (**Supplementary Figure 3**). This gender bias has not been addressed in the literature and will require further studies. The possible explanation for these findings may be related to the phenomenon that CP occurs more frequently in males (64) categorized as GMFCS V (65). Due to this limitation, these subjects require more advanced care, notably oral care and hygiene performed by the caregiver. Perhaps the females were able to perform oral hygiene on their own due to fewer neuromotor limitations, which in turn may have resulted in greater GB. Additionally, hormonal changes in puberty should be examined as a factor for this observed gender dichotomy (66).

Inflammatory biomarkers can be evaluated both in serum (67) or in saliva (8). Blood collection in subjects with CP is a hard task, because these subjects present sympathetic nervous system predominance (68), resulting in vasoconstriction, and consequently making peripheral venous access more difficult. On the other hand, saliva is readily available, and its components are easy to be collected, and that has been used to evaluate salivary parameters in subjects with CP (15), ideal for future longitudinal monitoring.

The comparison between the constipated and non-constipated subjects in this study showed that there was approximately a doubled average production of cytokines favoring the potentiation of inflammation in these individuals (**Figures 4A–E**). In the presence of dysbiosis, virulence factors are released from pathogenic microorganisms present in the oral cavity, activating host's immune-inflammatory responses (69–71). IL-1β is an inflammatory cytokine associated with innate immune response, inflammation, pathogenesis, and progression of periodontal disease (72, 73). Higher levels of IL-1β ($P < 0.05$) were found in this study's subjects, who were constipated and presented higher values of GB (**Figure 4A**, **Supplementary Figure 2A**). Previous studies showed higher levels of IL-1β, IL-6, IL-8 in spastic CP subjects presenting GMCFS level V (15). Significantly higher levels of IL-6, IL-8, and IL-10 were found in constipated subjects with GB (**Figures 4C–E**; **Supplementary Figures 2C–E**). These cytokines represent the action of pro-inflammatory and anti-inflammatory cytokines (73), critical biomarkers of periodontal inflammation corresponding to the clinical severity of the disease (56). IL-6 and IL-8 were also described as potential predictors for oral diseases, reinforcing the importance of evaluating the salivary levels of these biomarkers (74). The presence of the chemokine IL-8 induces the secretion of lymphocytes, monocytes, epithelial cells, fibroblasts, tumor cells, bone resorption, and IL-1β, indicating an ongoing inflammatory process and the progression of periodontal disease (57, 58). IL-10 acts as an anti-inflammatory cytokine, inhibiting pro-inflammatory cytokines IL-1 and IL-6 associated with improvements in periodontal clinical parameters (28). However, the degree of mucosal inflammation of constipated CP subjects represented by GB was so high that IL-10 action did not reduce the inflammatory process of dysbiosis (59). Only one study reported the effect of mechanical treatment on gingivitis control measures by cytokines levels, and the results showed that, although reduction of inflammatory process occurs, the levels of IL-1β, IL-6, and IL-8 remain high compared to subjects without CP (8).

These results highlight that prediction of systemic health in CP patients by salivary cytokine monitoring is a reliable approach, yet this field is in its infancy. Novel methodological assays to control for time, type of salivary collection and flow on a longitudinal basis would provide more reliable findings, especially accounting for inter- and intra-individual variations. We were able to demonstrate that not only were there significant differences between gut constipation and oral inflammation with specificity for the type of cytokines, we also demonstrated that specific cytokines significantly correlated with the type of medication. CP inaccessibility and vulnerability to oral care, and consequent development of caries and gum diseases may also impact gut phenotypically via microbiome oral-gut axis. These data does not *per se* indicate microbiome changes, but

indicates that gingival bleeding index suggests dysbiosis in host-microbial interactions at the oral mucosal interface, which can, in turn, influence an individual's systemic inflammatory profile. New studies should be developed addressing inflammatory salivary cytokines, oral pathogens correlated with intestinal constipation. While our investigations demonstrated how salivary inflammatory cytokines specifically correlated with gut constipation and quality of life, and it was well-controlled for gender statistical power, limitations were also noted. Dental plaque levels were not recorded. This is an important focus of future studies, specifically dissecting to oral microbes that are modulated by CP environment and medication. We have included gingival bleeding as surrogate to oral host responses to the presence of the biofilm. In future examinations, we aim to look into the multimodal relationship of salivary cytokines, gut constipation as well as gingival bleeding with oral hygiene and dental plaque levels.

In conclusion, the results suggest that the higher levels of inflammatory markers, present in saliva, directly correlated with reduced quality of life, due to excessive gut constipation. Thus, increase of inflammation and gut constipation were directly correlated, impacting quality of life. These are novel relationships in investigating oral-gut-brain axis in investigating the clinical impact of inflammation throughout the human body, and providing reliable information through non-invasive salivary diagnostics.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of the Cruzeiro do Sul University-Brazil Platform, São Paulo, Brazil (IRB #2,452,626). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AF, MS, and MF: conceived and designed the experiments and wrote the paper. AF: performed the experiments. AF and RE: analyzed the data. RE: prepared graphs. MF: project administration. All authors: reviewed the manuscript.

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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.2021.619262/full#supplementary-material

Supplementary Figure 1 | Effects of CPCHILD Subscores on Overall QOL. Distributions of each individual CPCHILD subscore **(A)** Personal Care; **(B)** Positioning, Transferring, and Mobility; **(C)** Communication and Social Interaction; **(D)** Comfort, Emotions, and Behaviors; **(E)** and Health is measured on the x-axes, while the y-axis marks the average of the remaining subscores. Lines show a trendline of data sets with shaded regions marking the 95% confidence interval ($n = 93$).

Supplementary Figure 2 | Gingival Bleeding association with specific cytokines. Scatterplots of collected cytokine levels —**(A)** IL-1β, **(B)** TNF-α, **C)** IL-6, **(D)** IL-8, and **(E)** IL-10—and Gingival Bleeding percentage. Blue line marks the linearly regressed trendline (lm) and the shaded region corresponds to the 95% confidence interval. *P*-values from ANOVA are: IL-1β, $P < 0.0001$—TNF-α, $P = 0.237$—IL-6, $P < 0.05$—IL-8, $P < 0.01$—IL-10, $P < 0.01$. **(F)** Standard box plot comparing AED used and Gingival Bleeding Percentage. AED used is significantly correlated with GB ($P < 0.0001$), $n = 63$, significance was evaluated by ANCOVA, p-value < 0.05. Furthermore, both intramedication t-tests were employed in addition to Bonferroni-corrected intermedication Welch's *t*-tests (only constipated subjects) for each medication as compared to control (Key: ns - $p \geq 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Supplementary Figure 3 | Factors impacting gingival bleeding. **(A)** Violin plots depicting gingival bleeding patterns stratified by gender. Box plot within the violin represent the interquartile range, and whiskers show the largest outliers within 150% the interquartile range above the 75th percentile and below the 25th percentile. **(B)** Scatter plot demonstrating gingival bleeding in relation to age distribution. Blue line marks trendline, where the shaded region marks the 95% confidence interval. **(C)** Gingival bleeding plotted by Cerebral Palsy Diagnosis. Boxes represent interquartile range, center line represents the median, while whiskers represent 95% confidence interval. The box's color notates the gender, with female being orange and male being blue ($n = 93$). For **(A,C)**, Welch's *t*-tests were employed along gender lines (Key: ns – $p \geq 0.05$, $^{*}p < 0.05$).

REFERENCES

1. Rosenbaum P, Paneth N, Leviton A, Goldstein M, Bax M, Damiano D, et al. A report: the definition and classification of cerebral palsy April (2006). *Dev Med Child Neurol Suppl.* (2007) 109:8–14.
2. Nelson KB, Dambrosia JM, Grether JK, Phillips TM. Neonatal cytokines and coagulation factors in children with cerebral palsy. *Ann Neurol.* (1998) 44:665–75. doi: 10.1002/ana.410440413
3. Maenner MJ, Blumberg SJ, Kogan MD, Christensen D, Yeargin-Allsopp M, Schieve LA. Prevalence of cerebral palsy and intellectual disability among children identified in two U.S. National Surveys, 2011-2013. *Ann Epidemiol.* (2016) 26:222–6. doi: 10.1016/j.annepidem.2016.01.001
4. Wimalasundera N, Stevenson VL. Cerebral palsy. *Pract Neurol.* (2016) 16:184–94. doi: 10.1136/practneurol-2015-001184
5. Sewell MD, Eastwood DM, Wimalasundera N. Managing common symptoms of cerebral palsy in children. *BMJ.* (2014) 349:g5474. doi: 10.1136/bmj.g5474

6. Palisano R, Rosenbaum P, Walter S, Russell D, Wood E, Galuppi B. Development and reliability of a system to classify gross motor function in children with cerebral palsy. *Dev Med Child Neurol.* (1997) 39:214–23. doi: 10.1111/j.1469-8749.1997.tb07414.x
7. Ferreira ACFM, Mayer MPA, Kawamoto D, Santos MTBR. Constipation, antiepileptic drugs, and gingivitis in children and adolescents with cerebral palsy. *Int J Paediatr Dentist.* (2019) 29:635–41. doi: 10.1111/ipd.12488
8. Yoshida RA, Gorjão R, Mayer MPA, Corazza PFL, Guare RO, Fereeira ACFM, et al. Inflammatory markers in the saliva of cerebral palsy individuals with gingivitis after periodontal treatment. *Braz Oral Res.* (2019) 33:E033. doi: 10.1590/1807-3107bor-2019.vol33.0033
9. Gutierrez GM, Siqueira VL, Loyola-Rodriguez JP, Diniz MB, Guaré RO, Ferreira ACFM, et al. Effects of treatments for drooling on caries risk in children and adolescents with cerebral palsy. *Med Oral Patol Oral Cir Bucal.* (2019) 24:204–10. doi: 10.4317/medoral.22729
10. Delacy MJ, Reid SM. Profile of associated impairments at age 5 years in Australia by cerebral palsy subtype and gross motor function classification system level for birth years 1996 to 2005. *Med Child.* (2016) 58:50–6. doi: 10.1111/dmcn.13012
11. Veugelers R, Benninga MA, Calis EAC, Willemsen SP, Evenhuis H, Tibboel D, et al. Prevalence and clinical presentation of constipation in children with severe generalized cerebral palsy. *Dev Med Child Neurol.* (2010) 52:e216–21. doi: 10.1111/j.1469-8749.2010.03701.x
12. Johanson JF, Sonnenberg A, Koch TR, McCarty DJ. Association of constipation with neurologic diseases. *Dig Dis Sci.* (1992) 37:179–86. doi: 10.1007/BF01308169
13. Park ES, Park CI, Cho SR, Na SI, Cho YS. Colonic transit time and constipation in children with spastic cerebral palsy. *Arch Phys Med Rehabil.* (2004) 85:453–6. doi: 10.1016/S0003-9993(03)00479-9
14. Jahromi SR, Togha M, Fesharaki SH, Najafi M, Moghadam NB, Kheradmand JA, et al. Gastrointestinal adverse effects of antiepileptic drugs in intractable epileptic patients. *Seizure.* (2011) 20:343–6. doi: 10.1016/j.seizure.2010.12.011
15. Santos MTBR, Diniz MB, Guaré RO, Ferreira MCD, Gutierrez GM, et al. Inflammatory markers in saliva as indicators of gingival inflammation in cerebral palsy children with and without cervical motor control. *Int J Paediatr Dentist.* (2017) 27:364–71. doi: 10.1111/ipd.12270
16. Bensi C, Costacurta M, Docimo R. Oral health in children with cerebral palsy: a systematic review and meta-analysis. *Spec Care Dentist.* (2020) 40:401–11. doi: 10.1111/scd.12506
17. Gunel MK, Mutlu A, Tarsuslu T, Livanelioglu A. Relationship among the manual ability classification system (MACS), the gross motor function classification system (GMFCS), and the functional status (WeeFIM) in children with spastic cerebral palsy. *Eur J Pediatr.* (2009) 168:477–85. doi: 10.1007/s00431-008-0775-1
18. Bearden DR, Monokwane B, Khurana E, Baier J, Baranov E, Westmoreland K, et al. Pediatric cerebral palsy in botswana: etiology, outcomes, and comorbidities. *Pediatr Neurol.* (2016) 59:23–9. doi: 10.1016/j.pediatrneurol.2016.03.002
19. Selai C, Bannister D, Trimble M. Antiepileptic drugs and the regulation of mood and quality of life (QOL): the evidence from epilepsy. *Epilepsia.* (2005) 46 (Suppl. 4):50–7. doi: 10.1111/j.1528-1167.2005.46 3010.x
20. Dammann O, O'Shea TM. Cytokines and perinatal brain damage. *Clin Perinatol.* (2008) 35:643–63. doi: 10.1016/j.clp.2008.07.011
21. Malaeb S, Dammann O. Fetal inflammatory response and brain injury in the preterm newborn. *J Child Neurol.* (2009) 24:1119–26. doi: 10.1177/0883073809338066
22. Carlo WA, McDonald SA, Tyson JE, Stoll BJ, Ehrenkranz RA, Shankaran S, et al. Cytokines and neurodevelopmental outcomes in extremely low birth weight infants. *J Pediatr.* (2011) 159:919–25.e3. doi: 10.1016/j.jpeds.2011.05.042
23. Młodzikowska-Albrecht J, Steinborn B, Zarowski M. Cytokines, epilepsy and epileptic drugs–is there a mutual influence? *Pharmacol Rep.* (2007) 59:129–38.
24. Zhu H, Lin X, Zheng P, Chen H. Inflammatory cytokine levels in patients with periodontitis and/or coronary heart disease. *Int J Clin Exp Pathol.* (2015) 8:2214–20.
25. Gomes FIF, Aragão MGB, Barbosa FCB, Bezerra MM, de Paulo TPV, Chaves HV. Inflammatory cytokines interleukin-1β and tumour necrosis factor-α - novel biomarkers for the detection of periodontal diseases: a literature review. *J Oral Maxillofac Res.* (2016) 7:e2. doi: 10.5037/jomr.2016.7202
26. Kardeşler L, Buduneli N, Çetinkalp S, Lappin D, Kinane DF. Gingival crevicular fluid IL-6, tPA, PAI-2, albumin levels following initial periodontal treatment in chronic periodontitis patients with or without type 2 diabetes. *Inflamm Res.* (2011) 60:143–51. doi: 10.1007/s00011-010-0248-7
27. Zhang N, Xu Y, Zhang B, Zhang T, Yang H, Zhang B, et al. Analysis of interleukin-8 gene variants reveals their relative importance as genetic susceptibility factors for chronic periodontitis in the Han population. *PLoS ONE.* (2014) 9:e104436. doi: 10.1371/journal.pone.0104436
28. Zhang Q, Chen B, Yan F, Guo J, Zhu X, Ma S, et al. Interleukin-10 inhibits bone resorption: a potential therapeutic strategy in periodontitis and other bone loss diseases. *Biomed Res Int.* (2014) 2014:284836. doi: 10.1155/2014/284836
29. Verrotti A, Basciani F, Trotta D, Greco R, Morgese G, Chiarelli F. Effect of anticonvulsant drugs on interleukins-1,−2 and−6 and monocyte chemoattractant protein-1. *Clin Exp Med.* (2001) 1:133–6. doi: 10.1007/s10238-001-8024-1
30. Godhwani N, Bahna SL. Antiepilepsy drugs and the immune system. *Ann Allergy Asthma Immunol.* (2016) 117:634–40. doi: 10.1016/j.anai.2016.09.443
31. Pellegrino LA, Ortolan EVP, Magalhães CS, Viana AA, Narayanan UG. Brazilian Portuguese translation and cross-cultural adaptation of the "caregiver priorities and child health index of life with disabilities" (CPCHILD©) questionnaire. *BMC Pediatr.* (2014) 14:30. doi: 10.1186/1471-2431-14-30
32. Lewis SJ, Heaton KW. Stool form scale as a useful guide to intestinal transit time. *Scand J Gastroenterol.* (1997) 32:920–4. doi: 10.3109/00365529709011203
33. Jozala DR, de Faria Oliveira IS, Ortolan EVP, de Oliveira Junior WE, Comes GT, Cassettari VMG, et al. Brazilian Portuguese translation, cross-cultural adaptation and reproducibility assessment of the modified bristol stool form scale for children. *J Pediatr.* (2019) 95:321–7. doi: 10.1016/j.jped.2018.01.006
34. Martinez AP, de Azevedo GR. The bristol stool form scale: its translation to Portuguese, cultural adaptation and validation. *Rev Lat Am Enfermagem.* (2012) 20:583–9. doi: 10.1590/S0104-11692012000300021
35. Chaves ES, Wood RC, Jones AA, Newbold DA, Manwell MA, Kornman KS. Relationship of "bleeding on probing" and "gingival index bleeding" as clinical parameters of gingival inflammation. *J Clin Periodontol.* (1993) 20:139–43. doi: 10.1111/j.1600-051X.1993.tb00328.x
36. Chapple ILC, Mealey BL, Van Dyke TE, Bartold PM, Dommisch H, Eickholz P, et al. Periodontal health and gingival diseases and conditions on an intact and a reduced periodontium: consensus report of workgroup 1 of the 2017 world workshop on the classification of periodontal and peri-implant diseases and conditions. *J Periodontol.* (2018) 89:S74–84. doi: 10.1002/JPER.17-0719
37. Dean, AG. *Openepi : Open Source Epidemiologic Statistics for Public Health.* (2007) Available online at: https://ci.nii.ac.jp/naid/10025863040/ (accessed October 18, 2020).
38. Mugie SM, Benninga MA, Di Lorenzo C. Epidemiology of constipation in children and adults: a systematic review. *Best Pract Res Clin Gastroenterol.* (2011) 25:3–18. doi: 10.1016/j.bpg.2010.12.010
39. Pulgar S, Bains S, Gooch J, Chambers H, Noritz GH, Wright E, et al. Prevalence, patterns, and cost of care for children with cerebral palsy enrolled in medicaid managed care. *J Manag Care Spec Pharm.* (2019) 25:817–22. doi: 10.18553/jmcp.2019.25.7.817
40. Fiori S, Staudt M, Boyd RN, Guzzetta A. Neural plasticity after congenital brain lesions. *Neural Plast.* (2019) 2019:9154282. doi: 10.1155/2019/9154282
41. Braccialli LMP, Almeida VS, Sankako AN, Silva MZ, Braccialli AC, Carvalho SMR, et al. Translation and validation of the Brazilian version of the cerebral palsy quality of life questionnaire for children – child report. *J Pediatr.* (2016) 92:143–8. doi: 10.1016/j.jpedp.2015.12.005
42. Ickes SB, Hurst TE, Flax VL. Maternal literacy, facility birth, and education are positively associated with better infant and young child feeding practices and nutritional status among Ugandan children. *J Nutr.* (2015) 145:2578–86. doi: 10.3945/jn.115.214346
43. Liu HY, Chen JR, Hsiao SY, Huang ST. Caregivers' oral health knowledge, attitude and behavior toward their children with disabilities. *J Dent Sci.* (2017) 12:388–95. doi: 10.1016/j.jds.2017.05.003
44. Sullivan AB, Miller D. Who is taking care of the caregiver? *J Patient Exp.* (2015) 2:7–12. doi: 10.1177/237437431500200103

45. Barros ALO, de Gutierrez GM, Barros AO, Santos MTBR. Quality of life and burden of caregivers of children and adolescents with disabilities. *Spec Care Dentist.* (2019) 39:380–8. doi: 10.1111/scd.12400
46. Fairhurst C, Shortland A, Chandler S, Will E, Scrutton D, Simonoff E, et al. Factors associated with pain in adolescents with bilateral cerebral palsy. *Dev Med Child Neurol.* (2019) 61:929–36. doi: 10.1111/dmcn.14113
47. Tan SS, van Gorp M, Voorman JM, Geytenbeek JJ, Reinders-Messelink HA, Ketelaar M, et al. Development curves of communication and social interaction in individuals with cerebral palsy. *Dev Med Child Neurol.* (2020) 62:132–9. doi: 10.1111/dmcn.14351
48. Devinsky O, Vezzani A, O'Brien TJ, Jette N, Scheffer IE, de Curtis M, et al. Epilepsy. *Nat Rev Dis Prim.* (2018) 4:18024. doi: 10.1038/nrdp.2018.24
49. Kwan P, Arzimanoglou A, Berg AT, Brodie MJ, Allen Hauser W, Mathern G, et al. Definition of drug resistant epilepsy: consensus proposal by the ad hoc task force of the ILAE commission on therapeutic strategies. *Epilepsia.* (2009) 51:1069–77. doi: 10.1111/j.1528-1167.2009.02397.x
50. Blond BN, Detyniecki K, Hirsch LJ. Assessment of treatment side effects and quality of life in people with epilepsy. *Neurol Clin.* (2016) 34:395–410. doi: 10.1016/j.ncl.2015.11.002
51. Hyland NP, Cryan JF. A gut feeling about GABA: focus on GABA(B) receptors. *Front Pharmacol.* (2010) 1:124. doi: 10.3389/fphar.2010.00124
52. Sedky NA. Assessment of oral and dental health status in children with cerebral palsy: an exploratory study. *Int J Health Sci.* (2018) 12:4–14.
53. Dos Santos MTBR, Nogueira MLG. Infantile reflexes and their effects on dental caries and oral hygiene in cerebral palsy individuals. *J Oral Rehabil.* (2005) 32:880–5. doi: 10.1111/j.1365-2842.2005.01518.x
54. Ming X, Chen N, Ray C, Brewer G, Kornitzer J, Steer RA. A gut feeling: a hypothesis of the role of the microbiome in attention-deficit/hyperactivity disorders. *Child Neurol Open.* (2018) 5:2329048X18786799. doi: 10.1177/2329048X18786799
55. Finoti LS, Nepomuceno R, Pigossi SC, Corbi SC, Secolin R, Scarel-Caminaga RM. Association between interleukin-8 levels and chronic periodontal disease: a PRISMA-compliant systematic review and meta-analysis. *Medicine.* (2017) 96:e6932. doi: 10.1097/MD.0000000000006932
56. Batool H, Nadeem A, Kashif M, Shahzad F, Tahir R, Afzal N. Salivary levels of IL-6 and IL-17 could be an indicator of disease severity in patients with calculus associated chronic periodontitis. *Biomed Res Int.* (2018) 2018:8531961. doi: 10.1155/2018/8531961
57. Ertugrul AS, Sahin H, Dikilitas A, Alpaslan N, Bozoglan A. Comparison of CCL28, interleukin-8, interleukin-1β and tumor necrosis factor-alpha in subjects with gingivitis, chronic periodontitis and generalized aggressive periodontitis. *J Periodont Res.* (2013) 48:44–51. doi: 10.1111/j.1600-0765.2012.01500.x
58. Gamonal J, Acevedo A, Bascones A, Jorge O, Silva A. Characterization of cellular infiltrate, detection of chemokine receptor CCR5 and interleukin-8 and RANTES chemokines in adult periodontitis. *J Periodont Res.* (2001) 36:194–203. doi: 10.1034/j.1600-0765.2001.360309.x
59. Costa FO, Cortelli SC, Silva TA, Costa AA, Lima RPE, Cortelli JR, et al. Cytokine levels in crevicular fluid associated with compliance during periodontal maintenance therapy. *Clin Oral Investig.* (2019) 23:3517–26. doi: 10.1007/s00784-018-2770-x
60. Zhu L, Liu W, Alkhouri R, Baker RD, Bard JE, Quigley EM, et al. Structural changes in the gut microbiome of constipated patients. *Physiol Genom.* (2014) 46:679–86. doi: 10.1152/physiolgenomics.00082.2014
61. Khalif IL, Quigley EMM, Konovitch EA, Maximova ID. Alterations in the colonic flora and intestinal permeability and evidence of immune activation in chronic constipation. *Dig Liver Dis.* (2005) 37:838–49. doi: 10.1016/j.dld.2005.06.008
62. Li Q, Han Y, Dy ABC, Hagerman RJ. The gut microbiota and autism spectrum disorders. *Front Cell Neurosci.* (2017) 11:120. doi: 10.3389/fncel.2017.00120
63. Jyonouchi H, Sun S, Le H. Proinflammatory and regulatory cytokine production associated with innate and adaptive immune responses in children with autism spectrum disorders and developmental regression. *J Neuroimmunol.* (2001) 120:170–9. doi: 10.1016/S0165-5728(01)00421-0
64. Romeo DM, Sini F, Brogna C, Albamonte E, Ricci D, Mercuri E. Sex differences in cerebral palsy on neuromotor outcome: a critical review. *Dev Med Child Neurol.* (2016) 58:809–13. doi: 10.1111/dmcn.13137
65. Chen M, Li T, Lin S, Bi D, Zhu D, Shang Q, et al. Association of Interleukin 6 gene polymorphisms with genetic susceptibilities to spastic tetraplegia in males: a case-control study. *Cytokine.* (2013) 61:826–30. doi: 10.1016/j.cyto.2013.01.011
66. Nakagawa S, Fujii H, Machida Y, Okuda K. A longitudinal study from prepuberty to puberty of gingivitis. Correlation between the occurrence of Prevotella intermedia and sex hormones. *J Clin Periodontol.* (1994) 21:658–65. doi: 10.1111/j.1600-051X.1994.tb00783.x
67. Hu L, Mauro TM, Dang E, Man G, Zhang J, Lee D, et al. Epidermal dysfunction leads to an age-associated increase in levels of serum inflammatory cytokines. *J Invest Dermatol.* (2017) 137:1277–85. doi: 10.1016/j.jid.2017.01.007
68. Ferreira MC, Pastore C, Imada R, Guaré R, Leite M, Poyares D, et al. Autonomic nervous system in individuals with cerebral palsy: a controlled study. *J Oral Pathol Med.* (2011) 40:576–81. doi: 10.1111/j.1600-0714.2011.01008.x
69. Meyle J, Chapple I. Molecular aspects of the pathogenesis of periodontitis. *Periodontol 2000.* (2015) 69:7–17. doi: 10.1111/prd.12104
70. Huang N, Gibson FC 3rd. Immuno-pathogenesis of Periodontal Disease: Current and Emerging Paradigms. *Curr Oral Health Rep.* (2014) 1:124–32. doi: 10.1007/s40496-014-0017-8
71. Cekici A, Kantarci A, Hasturk H, Van Dyke TE. Inflammatory and immune pathways in the pathogenesis of periodontal disease. *Periodontology 2000.* (2014) 64:57–80. doi: 10.1111/prd.12002
72. Rudick CP, Lang MS, Miyamoto T. Understanding the pathophysiology behind chairside diagnostics and genetic testing for IL-1 and IL-6. *Oral Dis.* (2019) 25:1879–85. doi: 10.1111/odi.13030
73. Degasperi GR, Etchegaray A, Marcelino L, Sicard A, Villalpando K, Pinheiro SL. Periodontal disease: general aspects from biofilm to the immune response driven by periodontal pathogens. *Adv Microbiol.* (2018) 8:1. doi: 10.4236/aim.2018.81001
74. Rezaei F, Mozaffari HR, Tavasoli J, Zavattaro E, Imani MM, Sadeghi M. Evaluation of serum and salivary interleukin-6 and interleukin-8 levels in oral squamous cell carcinoma patients: systematic review and meta-analysis. *J Interferon Cytokine Res.* (2019) 39:727–39. doi: 10.1089/jir.2019.0070

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REVIEW
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The Role of the Immune Response in the Development of Medication-Related Osteonecrosis of the Jaw

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Medication-related osteonecrosis of the jaw (MRONJ) is a rare but serious adverse drug effect. There are multiple hypotheses to explain the development of MRONJ. Reduced bone remodeling and infection or inflammation are considered central to the pathogenesis of MRONJ. In recent years, increasing evidence has shown that bisphosphonates (BPs)-mediated immunity dysfunction is associated with the pathophysiology of MRONJ. In a healthy state, mucosal immunity provides the first line of protection against pathogens and oral mucosal immune cells defense against potentially invading pathogens by mediating the generation of protective immunoinflammatory responses. In addition, the immune system takes part in the process of bone remodeling and tissue repair. However, the treatment of BPs disturbs the mucosal and osteo immune homeostasis and thus impairs the body's ability to resist infection and repair from injury, thereby adding to the development of MRONJ. Here, we present the current knowledge about immunity dysfunction to shed light on the role of local immune disorder in the development of MRONJ.

Keywords: jaw, bisphosphonates, osteonecrosis, immunity, inflammation

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INTRODUCTION

Medication-related osteonecrosis of the jaw (MRONJ) is a severe medication-induced necrotic bone disease (1). According to the definition by the American Association of Oral and Maxillofacial Surgeons (AAOMS) in 2014, MRONJ is characterized by the following: "(1) exposed bone in the maxillofacial region that does not heal within 8 weeks after identification; (2) exposure to an anti-resorptive agent; and (3) no history of radiation therapy to the craniofacial region" (2). However, based on newest recommendations (2020) on MRONJ, MRONJ is defined as an "adverse drug reaction described as the progressive destruction and death of bone that affects the mandible and maxilla of patients exposed to the treatment with medications known to increase the risk of disease, in the absence of a previous radiation treatment" (3). Additionally, the workshop of the European task

force on MRONJ in 2019 suggested that it may no longer be necessary to require an 8-week observation of potential MRONJ manifestation to fit the case definition (4).

MRONJ was first found in patients receiving anti-resorptive therapy (bisphosphonates) and has not only affected the patients' quality of life but also interfered with the decision-making process of most dentists. Exposure to anti-resorptive medication was identified as a main risk factor of MRONJ. Although dosage and duration of administration were considered to be related to the occurrence of MRONJ (5, 6), recent clinical data from a 14-year retrospective survey showed that the number of patients treated with low-dose anti-resorptive medication accounted for over half of the MRONJ cases (7). In addition, some potential risk factors, including periodontal diseases, tooth extraction or dental implantation, and chemotherapy were reported to be associated with the occurrence of MRONJ (8–11). With aging, the number of people with skeletal-related events (SREs) who need anti-resorptive treatment increases. To balance the risks and benefits of these medications, it is imperative to explore the pathophysiology of MRONJ.

Now, there has been an increase in new kinds of medication that have been reported to induce the occurrence of MRONJ, such as another anti-resorptive medication, a monoclonal antibody against the receptor activator of nuclear factor-κB (RANK) ligand (RANKL): denosumab and vascular endothelial growth factor inhibitors (12): bevacizumab, Sunitinib. The mechanisms of MRONJ differ from medication to medication. The long-term administration of bisphosphonates and denosumab will cause disturbed remodeling due to their effect on osteoclast differentiation. Whereas, bevacizumab and Sunitinib- induced MRONJ may be associated with inhibition of angiogenesis. Currently, findings regarding MRONJ pathogenesis were categorized into several hypotheses, including disturbed bone remodeling, inflammation or infection, altered immunity, soft tissue toxicity, and inhibition of angiogenesis (13). Although accumulating evidence has shown that immunity dysfunction is highly involved in the development of MRONJ (14), there are few reports to systematically demonstrate the role of altered immunity in the development of MRONJ. This review highlights the current knowledge regarding the role of immunity dysfunction in the development of MRONJ, including mucosal and osteo immune responses.

THE MUCOSAL IMMUNE RESPONSE AND MRONJ

The mucosal immune system defenses against a variety of microbes and maintains the immune homeostasis in the oral cavity under healthy conditions (15). It has a sophisticated anatomical structure, several indigenous microorganisms, and immune cells. A tightly interlaced cell-to-cell network of epithelial cells acts as a physical barrier, thereby defending external stimuli and balancing the intricate interaction between the host and exterior environment (16). Furthermore, several indigenous microorganisms act as a biological barrier to prevent pathogen colonization (17). Moreover, various immune cells and secreted inflammatory cytokines play an important role in immune surveillance and homeostasis (18, 19). Unfortunately, BPs and risk factors that impact the mucosal immune system, coupled with bacterial infection contribute to the development of MRONJ.

The Destruction of Mucosal Barrier Protection

There are hundreds of thousands of bacterial species in the oral cavity (20). An imbalanced bacterial flora can contribute to multiple mucosal diseases (21). Colonization of unique bacterial communities, coupled with a dysfunctional innate immunity, has been shown to affect the pathogenesis of ONJ (22). Indigenous microbiota, as biological barriers, are antagonistic bacteria, which suppress the invasion and colonization of harmful microorganisms. Several protective mechanisms of indigenous microbiota have been reported, including competition for nutrients, direct killing, and enhancement of immune responses (23). In a previous report, it was demonstrated that BPs exerted inhibitory effects on the growth of select bacterial species (24). Recently, Williams et al. demonstrated increased local bacterial infiltration in MRONJ mice (25). Moreover, after extracting healthy teeth from mice, and following with zoledronate infusions, no differences were observed in the formation of osteonecrosis between mice receiving broad-spectrum antibiotic treatment and negative controls. Interestingly, antibiotic-mediated oral dysbiosis causes increased bone necrosis when extracting teeth with ligature-induced periodontitis. Moreover, they showed that broad-spectrum antibiotic treatment suppressed the normal flora to protect against inflammation-induced osteonecrosis by dampening the formation of osteonecrosis and by activating osteoclasts (25). The results showed that the imbalance in oral flora may be a prerequisite for MRONJ and biological barriers are of great necessity to prevent the occurrence of MRONJ (**Figure 1**). Clinical data also supported the view by the evidence that about half of individuals diagnosed with MRONJ are multiple myeloma (MM) patients, who mostly underwent antibiotics treatment for a long time (26). Although antibiotic treatment has been shown to be effective for MRONJ, increased attention should be paid to patients receiving long-term antibiotics treatment.

Different from the intestinal mucosal barrier, the specific structure of the dento-gingival junction makes the oral mucosal barrier more fragile. The bone-invasion dental measurement and mechanical trauma disrupt the integrity of the oral mucosal epithelial barrier and thus facilitate bacterial invasion and colonization, resulting in jawbone infection. Additionally, mucosal ulceration and periodontal diseases can have a destructive effect on the oral mucosal barrier, and mucosal ulceration is believed to be the initial pathologic event of ONJ (27). BPs have a direct toxic effect on soft tissue (28, 29). Keratinocytes and fibroblasts are two of the most important compartments in the mucosal barrier. Previous reports have shown that BPs can induce senescence of human oral keratinocytes (30) and suppress the cell viability and migration of keratinocytes and fibroblasts (31). Furthermore, high levels

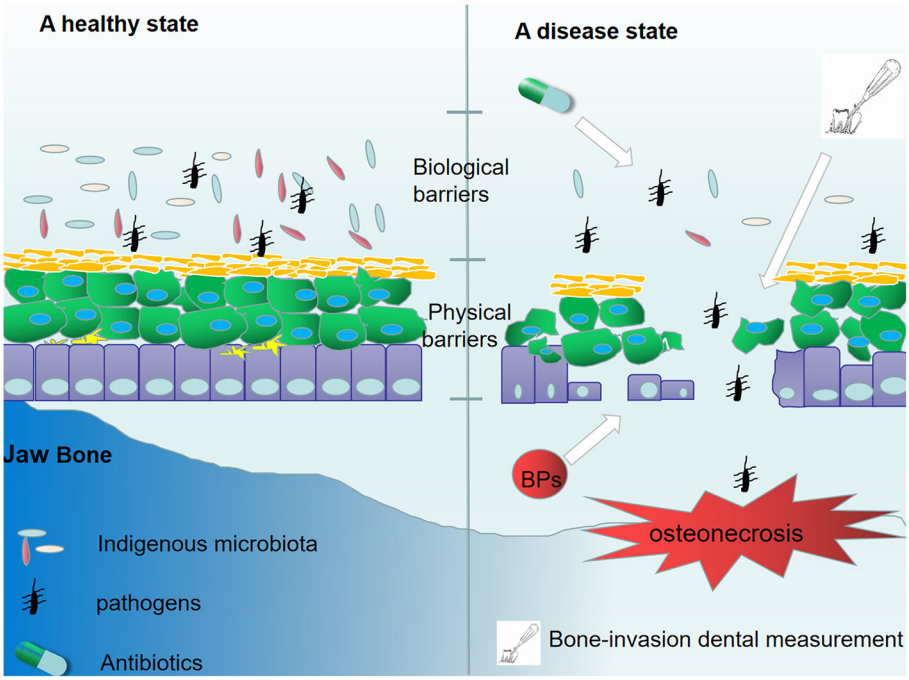

FIGURE 1 | The destruction of mucosal barrier protection. In a healthy state, indigenous microbiota and epithelial cells suppressed the invasion of pathogens. However, the long-time administration of antibiotics disturbs the oral normal flora, leading to a loss of biological barriers. Additionally, treatment with BPs and some bone-invasion dental measurements destroy the physical barrier composed by epithelial cells. The destruction of mucosal barrier protection facilitates bacterial invasion and colonization.

of bisphosphonates can cause apoptosis and necrosis (32). Therefore, disruption of the oral mucosal barrier is not only induced by potential risk factors but also by the toxic effect of BPs on soft tissue (**Figure 1**).

BPs-Mediated Dysfunction of Immune Cells and Unbalanced Inflammatory Cytokines

Once bacteria break down the mucosal barrier and invade, the hosts' innate immune response is triggered following inflammation to restrict pathogens and sustain homeostasis (33). Inflammation, a manifestation of the body against infection, is characterized by persistent infiltration of immune cells and enhanced levels of multiple pro-inflammatory cytokines and chemokines (34). Inflammation has been confirmed to be a pathological characteristic of MRONJ (35). In several mouse models of MRONJ and clinical ONJ cases, previous studies have shown increased bacterial infiltration and inflammation at the necrotic site (36). BPs, on the one hand, can induce an immunosuppressive condition by suppressing immune cell activation, on the other hand, it can render an imbalance between anti-inflammatory and pro-inflammatory cytokines, thereby resulting in intense inflammation and tissue damage.

Dendritic Cells

Dendritic cells (DCs), a type of antigen-presenting cell, play a vital role in initiating and regulating the immune response. Although a limited number of DCs (Langerhans cells) are present in the mucosal immune system, they can contact the external environment and regulate homeostasis of the oral immunity (37). Differentiated from hematopoietic stem cells, DCs are activated by antigens or other factors and present antigens to T cells. Moreover, DCs, as an essential link between innate and adaptive immune responses, are pivotal to maintain mucosal homeostasis (38). Dysfunctional DCs were found to induce an immunosuppressive condition in different diseases (39, 40). In a previous study, it was indicated that BPs exert inhibitory effects on DC activation, leading to immunosuppression or infectious complications (41). Orsini et al. demonstrated that BPs modulate the maturation of DCs but have no effect on the differentiation of DCs (42). Recently, it was shown that zoledronate (nitrogen-containing BPs) treatment decreased the number of infiltrated DCs in soft tissues and impaired the phagocytosis of DCs, thereby leading to an increased bacterial load and the occurrence of MRONJ (43). There are few reports on the underlying mechanism of BPs-mediated dysfunction of DCs, however, the immune suppression and inflammation in the oral cavity caused by dysfunctional DCs may provide new insights into the pathogenesis of MRONJ.

Macrophages

Macrophages are a type of heterogeneous mononuclear phagocytes positioned in different tissues, broadly including tissue-resident macrophages and infiltrating inflammatory

macrophages (44). Macrophages play key roles in immune surveillance and maintenance of the mucosal microenvironment homeostasis (45). In a clinical experimental study, a lower ratio of CD68/CD14 (CD14 and CD68 are markers of monocytes/macrophages) was observed in MRONJ lesions compared with other jaw infections, thereby indicating that macrophage immunosuppression induced by BPs may be linked to the development of MRONJ (46). Previously, tissue-resident macrophages were viewed as differentiated monocytes that migrated to tissues, however recently, the new view suggested that tissue-resident macrophages are locally being self-renewed (47). Due to the poor self-proliferative capability and short survival time, intestinal macrophages in mucosal immunity were believed to be derived from the constant recruitment of peripheral monocytes (48, 49). Therefore, macrophage migration is crucial for their role in mucosal immunity. However, macrophage migration and morphology were found to be altered by BPs in a dose-dependent manner (50). Lymphatic vessels and blood are required for the migration of immune cells. In a recent report, it was shown that BPs decreased the number of the larger-size $F4/80^{+}LYVE\text{-}1^{+}$ tube-like-structured cells (a type of macrophage) in impaired socket lesions with inhibition of lymphangiogenesis, which presented new immunopathological features in the MRONJ lesion (51). BPs were reported to suppress the viability of monocytic THP-1 cells after macrophage differentiation (52). The impaired migration and viability of macrophages may be associated with the development of MRONJ; however, there is little direct evidence about affecting the incidence of MRONJ.

The macrophage phenotype has been shown to play an important role in mediating inflammatory changes in tissues (53). Macrophages can be divided into two phenotypes (M1 and M2 macrophages) (54). Upon the stimulation of T-helper (T_H) 1 cytokines, such as IFN-γ and lipopolysccharide (LPS), macrophages are converted into M1 macrophages (55). M1 macrophages can produce pro-inflammatory cytokines, nitric oxide (NO), reactive oxygen species (ROS), interleukin (IL)-12, and tumor necrosis factor (TNF)-α, thereby playing an important role in host defense against pathogens. In addition, M2 macrophages are anti-inflammatory and serve an important role in eliminating inflammation and tissue modeling by secretion of IL-10 and TNF-β. M2 macrophages are polarized by stimulation of T_H2 cytokines, such as IL-4 or IL-13 (55). The balance of M1/M2 macrophage polarization maintains the immune homeostasis and governs the fate of organs. If the infection is serious enough to impair an organ, the M1 phenotype is exhibited to release pro-inflammatory cytokines against the stimulus. However, if the M1 phase persists, the tissue will be destroyed. Therefore, M2 macrophages release anti-inflammatory cytokines to suppress the inflammation and retain homeostasis (54). In recent studies, it was reported that imbalanced polarization of macrophages was associated with the pathogenesis of MRONJ (56). BPs were believed to enhance LPS-induced M1 macrophages through NLRP3 inflammasome activation, thereby amplifying inflammation by secreting pro-inflammatory cytokines (**Table 1**). Interestingly, it was further demonstrated that zoledronate did not have an effect on the differentiation of M2 macrophages in IL-4-treated human macrophages (THP-1 cells), and zoledronate did not pose an anti-inflammatory role via the polarization of M2 macrophages (65). Zhang et al. found that enhanced T-helper (T_H)17 cells coupled with IL-17 cytokines correlated with an elevated M1/M2 macrophages ratio at the local mucosal site of MRONJ lesion and that the inhibition of IL-17 activity reduced the elevated M1/M2 macrophages ratio as well as the incidence of MRONJ-like lesions in mice with multiple myeloma. Mechanistically, it was found that IL-17 can promote the polarization of M1 macrophages by activation of the STAT-1 signal pathway but inhibit M2 macrophage polarization by affecting IL-4-mediated activation of the STAT-6 signal pathway (61). In addition, Toll-like receptor family (TLRs)-mediated polarization of macrophages was reported to take part in the development of MRONJ. TLRs belong to pattern recognition receptors (PRRs). There are 12 TLR members. Some TLRs, including TLR-1,−2,−4,−5,−6, and−10, are cell membrane receptors, while others (TLR-3,−7,−8,−9,−11, and−13) are located within intracellular compartments (66). TLR-4 stands out in the polarization of macrophages (57). By inhibiting the mevalonate signaling pathway, zoledronate was reported to activate the TLR-4 signaling pathway and its downstream NF-κB signaling, which then enhanced M1 but inhibited M2 macrophage polarization. Furthermore, blocking the TLR-4 signaling pathway significantly inhibited the effect of zoledronate-mediated polarization of macrophages and decreased the number of M1 macrophages in the extraction socket tissues and the incidence of MRONJ (57). Therefore, TLR-4-mediated macrophage polarization and TLR-4 signaling might present novel therapeutic strategies for the treatment of patients with MRONJ (**Figure 2**). Taken together, the BPs-mediated alteration in M1/M2 macrophages in the mucosal immune system contributed to the pathogenesis of MRONJ.

γδT Cells

T cell receptors (TCRs) are unique antigen receptors of T cells (67). There are about 10^5 TCRs on the surface of a T cell. According to the different types of TCRs, T cells can be divided into αβT cells and γδT cells (68). It is well-known that αβT cells mainly participate in adaptive immunity, while γδT cells are involved in innate immunity. Moreover, γδT cells exist in the basal layer of oral mucosa and participate in protecting the integrity of the mucosal immune system. They also play a significant role in immunosurveillance, tissue repair, and homeostasis (69). Although BPs were believed to stimulate the expansion and cytotoxic activity of human γδT cells and play a role in cancer immunotherapy (70), in a previous study, it was shown that patients with MRONJ have a deficiency in circulating γδT cells in peripheral blood (71). Human γδ T cells are innate lymphocytes and are mainly composed of Vγ9Vδ2 T cells (71). BPs inhibit osteoclastic bone resorption by suppression of a key enzyme, farnesyl pyrophosphate synthase (FPPS), via the mevalonate pathway isoprenoid synthesis. As the natural substrate of FPPS, isopentenylpyrophosphate (IPP) is an endogenous antigen, which is recognized by a Vγ9Vδ2 T

TABLE 1 | The role of various cell cytokines in the development of MRONJ.

Cytokine	The role and function	Reference
IL-1	Pro-inflammatory factors / Delaying the wound healing	(57, 58)
IL-4	Anti-osteoclastogenic cytokines	(59)
IL-6	Osteoclastogenic cytokines	(59)
IL-10	Anti-inflammatory factor / Anti-osteoclastogenic cytokines	(59, 60)
IL-12	Pro-inflammatory factors	(60)
IL-17	Pro-inflammatory factors / T_H1 cytokines Osteoclastogenic cytokines	(57, 61) (59)
IL-23	Osteoclastogenic cytokines	(59)
IL-36	Pro-inflammatory factors	(62)
TNF-α	Pro-inflammatory factors	(60)
TNF-β	Anti-inflammatory factor	(60)
IFN-γ	Pro-inflammatory factors / Osteoclastogenic cytokines	(57, 59)
PDGF-BB	Pro-angiogenic and osteogenic cytokines	(63)
sSema4D	Pro-inflammatory factors	(64)

cell. The consequence of BPs-mediated inhibition of FPPS is the accumulation of cellular IPP, which results in Vγ9Vδ2 T cell stimulation in individuals receiving BPs therapy. The repeated activation of Vγ9Vδ2 T cells eventually causes γδ T cell deficiency (72). The loss of Vγ9Vδ2 T cells induced by BPs is defined as activation-induced cell death (73), which is a relatively common regulatory mechanism induced by the immune system to maintain homeostasis (74). Recently, Kalyan et al. found that BPs-treated neutrophils inhibited Vγ9Vδ2 T cell proliferation and that neutrophil-mediated inhibition of γδ T cell expansion could be rescued by suppressing neutrophil-derived hydrogen peroxide, serine proteases, and arginase I activity (75). These findings showed that treatment with BPs has an inhibitory effect on γδ T cells by affecting the function of neutrophils. These results can present reasons for BP-induced γδT cell deficiency, an underlying susceptibility to the development of MRONJ. However, in a recent report, Alexandru, et al. found increased infiltration of γδT cells and elevated expression of soluble semaphorin 4D (sSema4D) in MRONJ lesions whereas γδT cells only accounted for 2–5% of lymphocytes in the blood. SSema4D produced by γδT cells is involved in eliciting inflammation by promoting macrophages to produce pro-inflammatory cytokines, such as TNF-α, IL-1β, and IFN-γ. Consistently, the ablation of γδT cells in mice or neutralization of Sema4D by anti-Sema4D-mAb significantly decreased the incidence of MRONJ (64). Thus, γδT cells may likely be the source of Th1 cytokines, which provides insight into the pathogenesis of MRONJ. However, some researchers observed the incidence of ONJ-like lesions was lower in BPs-treated γδT null cells compared to wild mice. Human-γδT cells were injected to BPs-treated immunodeficient mice, and resulted in oral epithelial hyperplasia, but not osteonecrosis (73). These results illustrated that γδT cells are unlikely to affect the core osteonecrosis mechanism but may serve as an important modifier contributing to the occurrence of ONJ.

Neutrophil Granulocytes

Neutrophil granulocytes mainly exist in peripheral blood and take part in the early stage of immune responses (76). Neutrophil granulocytes, lymphocytes, and macrophages arise from hematopoietic stem cells and can differentiate into osteoclasts. Neutrophils are innate immune cells possessing phagocytotic ability and exert immune effects via activation of multiple effector responses and the generation of large amounts of reactive oxygen species (ROS) (77). These cells are also the targets of BPs. Hence, these cells might be of utmost importance in the etiology of MRONJ. Indeed, BPs have been shown to have a pro-inflammatory effect by affecting neutrophil function (78). Additionally, in a study regarding the immune cellular profile, it was shown that the number of polymorphonuclear neutrophils was increased in rats treated with BPs as well as several pro-inflammatory cytokines such as TNF-α and IL-1β, inducible nitric oxide synthase (iNOS), nuclear factor-kappa B (NF-kβ), and IL-18 binding protein (IL-18 bp) (79). These results indicated that BPs-altered neutrophils could exacerbate local inflammatory responses. However, Kuiper et al. suggested that exposure to BPs inhibited neutrophil chemotaxis, neutrophil NADPH oxidase activity, and decreased circulating neutrophils. The recruitment of neutrophils was also confirmed to be suppressed *in vivo*. Although the *in vitro* differentiation of neutrophils was not affected, their life span was shortened by BPs. Together, these results suggested that BPs have the potential to suppress the innate immune system, which possibly contributed to the pathogenesis of MRONJ (80). Moreover, a compromise in neutrophil function induced by BPs may be a potential biomarker for MRONJ susceptibility (81). The BPs-altered neutrophil functional defects may shed light on the role of local immunity in the development of MRONJ.

The Mucosal Immune Response and the Impaired Wound Healing

MRONJ is characterized by the delayed epithelial wound healing and exposed necrotic bone (1). The mucosal immune system exerts an important effect on controlling infection, as well as wound healing. Keratinocytes and fibroblasts are two of the most important components in the mucosal barrier. High viability of keratinocytes and fibroblasts is essential for epithelial wound healing. BPs have been reported to have a direct toxic effect on soft tissue (82). In addition to the direct toxic effect, BPs also exert an inhibitory effect on wound healing by altering immune cytokines (83). In a previous report, it was demonstrated that higher concentrations of anti-resorptives impaired wound healing accompanied by increased levels of inflammatory cytokines (84). IL-36α, a pro-inflammatory cytokine, is released by epithelial cells or innate immune cells and inhibits TGFβ-mediated collagen expression by activating the extracellular signal-regulated kinase (ERK) signal pathway in mouse gingival mesenchymal stem cells (62). A notably enhanced level of IL-36α and decreased level of collagen were observed in MRONJ lesions. Inhibition of the IL-36α pathway in mice alleviated

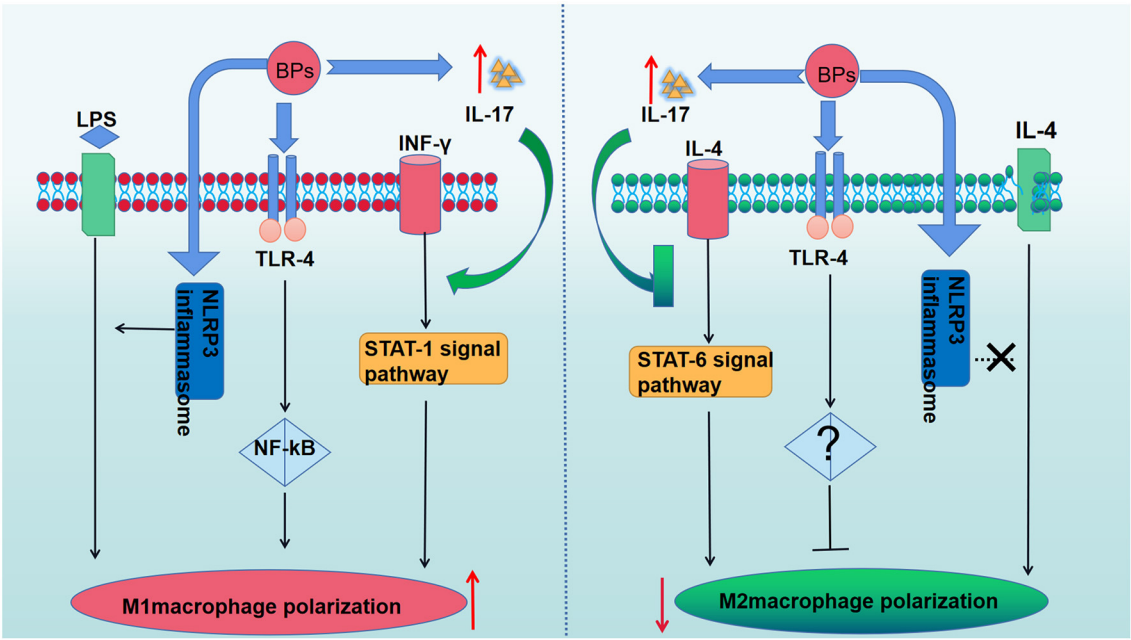

FIGURE 2 | The role of BPs in the macrophage polarization. BPs increase M1 macrophages polarization by activating the TLR-4 signaling pathway and its downstream NF-κB signaling pathway. The polarization of M2 macrophages was suppressed due to the activation of the TLR-4 signaling pathway. BPs enhance LPS-induced M1 macrophages through NLRP3 inflammasome activation but have no effect on the differentiation of M2 macrophages in IL-4-treated human macrophages. The enhanced IL-17 can promote the polarization of M1 macrophages by activation of the STAT-1 signal pathway but inhibit M2 macrophages polarization by affecting IL-4-mediated activation of the STAT-6 signal pathway.

the development of MRONJ lesions by rescuing the expression of collagen (62). Zhang et al. demonstrated that BPs-mediated activation of pyrin domain-containing protein 3 (NLRP3) in macrophages delayed oral wound healing and contributed to MRONJ-like lesions by the secretion of IL-1β in diabetic mice. Mechanistically, it was found that supplementation with intermediate metabolites of the mevalonate pathway robustly abolished the promotion of BPs on the release of IL-1β from macrophages in response to NLRP3 activation. Together, these results suggested that BP-mediated immune-inflammatory responses impaired socket wound healing and made oral wound susceptible to the development of MRONJ (58). In addition, dynamic polarization shifting from M1 to M2 macrophages plays an important role in wound healing (85). Recently, epidermal growth factor (EGF) was reported to partially rescue the effects of BPs on human oral keratinocytes (HOKs) and human umbilical vein endothelial cells (HUVECs) via the EGFR/AKT/PI3K signaling pathway *in vitro* (86). Additionally, exposure to BPs and bacterial infection at tooth extraction sites decreases the expression of keratinocyte growth factor (KGF), an epithelial cell-specific growth and differentiation factor, and, in turn, the reduction of KGF delays the epithelial wound-healing process, thereby contributing to the development of MRONJ (87). Impaired recruitment of endothelial progenitor cells (EPCs) by BPs was also considered an etiological factor of delayed wound healing. Local injection of EPCs stimulated the healing of MRONJ-like lesions by increasing vascularization and improving epithelial and fibroblast functions (88). However, Yamashita et al. suggested that BPs have an effect on impaired osseous wound healing by decreasing the expression of VEGF-C and MMP-13 but was not associated with angiogenic markers (CD31 and VEGF-A) in the bone marrow or soft tissue wound healing (89). In other words, zoledronate selectively suppressed bone healing but did not influence soft tissue healing in the oral cavity. Osseous wound healing and soft tissue wound healing are two important stages of bone necrosis healing that need further exploration.

THE OSTEO IMMUNE RESPONSE AND MRONJ

Bone is dynamic tissue undergoing continuous remodeling and repair to maintain bone homeostasis (90). Bone remodeling is a process, which is related to osteoblasts-mediated osteogenesis and osteoclasts-mediated osteoclastogenesis. During the healthy state, bone resorption and bone formation take place at equal rates to ensure an adequate quantity of bone mass and a healthy functional status (91). However, inflammatory diseases are always accompanied by bone loss in the oral cavity, such as periodontitis (92). Under the inflammatory condition, the immune system not only protects against the invasive pathogens and reduces inflammation, but also maintains the bone homeostasis by removing the damaged and apoptotic tissue and stimulating the bone tissue repair and regeneration, which fostered a novel interdisciplinary field, "osteoimmunology"

(93). Moreover, many regulatory molecules are shared by the immune and skeletal system, including cytokines, receptors, signaling molecules, and transcription factors. Additionally, decades of reports have identified that immune cells participate and mediate skeletal homeostasis by releasing multiple types of cytokines (94). Elsayed et al. found that DCs play a significant role in post-extraction homeostasis in alveolar bone (43). Osteal macrophages were reported to locate adjacent to osteoblasts and regulate bone formation, playing diverse roles in skeletal homeostasis (95). However, BPs affect the process of immune and bone interaction, thereby leading to the imbalance of bone homeostasis (96, 97) and low bone turnover. The low bone turnover promotes the accumulation of microdamage in the jaw bone and the opportunity of bacterial colonization, being suggested as a contributor to the development of MRONJ (98). Therefore, understanding the role of BPs-altered osteo response in the process of bone remodeling is critical to our exploration of the pathogenesis of MRONJ.

RANK/RANKL/OPG Signaling Pathway and Osteoclastogenesis

RANKL, as one of the important molecules, links bone metabolism and immune response. Initially, RANKL was considered an activator of DCs released by activated T cells (99). Recently, in several reports, it was shown that RANKL could induce the differentiation of osteoclast precursors by binding to RANK, a type I membrane protein (100). Osteoprotegerin (OPG) has an inhibitory effect on the function of RANKL by binding to RANKL and preventing its binding to RANK (100). The relative ratio between RANKL and OPG plays an important role in initiating and maintaining osteoclastogenesis, thereby, resulting in bone resorption by osteoclasts, which is central to the remodeling of the jawbone (101). However, Nisio et al. found the increased expression of RANK, RANKL in BPs induced necrotic bone lesions, suggesting that colonizing bacteria that produced lipopolysaccharide could trigger the RANK/RANKL/OPG signaling pathway and enhance osteoclast differentiation and activation (102). It was suggested that osteoclast activation was a protective strategy from the host bone tissue to eliminate the necrotic area and infection.

In a previous report, it was illustrated that BPs modulate the expression of RANK, RANKL, and OPG, thus decreasing osteoclast activity (103). Moreover, BPs were confirmed to inhibit RANKL-induced osteoclast differentiation by the suppression of the nuclear factor of activated T-cells c1 (NFATc1) (104). As a transcription factor of the nuclear factor in the activated T cell (NFAT) family (105), NFATc1 is a vital downstream target of RANK. When RANKL binds to RANK, TNF receptor-associated factor 6 (Traf6) is recruited and activated, thereby causing signal pathway activation of NF-κB, MAPK, and c-Fos (106). These signal pathways trigger the activation of NFATc1 as well as the process of osteoclast differentiation. These results demonstrated that BPs impaired bone homeostasis by affecting the RANKL/OPG/RANK signaling pathway (**Figure 3**).

Cytokines and Osteoclastogenesis

Immune cells not only have the capacity to eliminate inflammation but also affect osteoclast differentiation by the release of multiple cytokines, including osteoclastogenic and anti-osteoclastogenic cytokines (107). Osteoclastogenic cytokines, such as TNF-α, IL-1, IL-6, IL-7, IL-8, IL-11, IL-15, IL-17, IL-23, and IL-34, promote osteoclast differentiation, while anti-osteoclastogenic cytokines have an inhibitory effect on osteoclast differentiation, including IFN-α, IFN-β, IFN-γ, IL-3, IL-4, IL-10, IL-12, IL-27, and IL-33. Clinical data showed that BPs-treated osteoporotic patients have significantly decreased levels of IL-6, IL-17, IL-23, and IFN-γ and a significant increase in IL-4, IL-10, and TGF-β compared to osteoporotic patients who did not receive BPs treatment. These results demonstrated that BPs have an inhibitory effect on osteoclast differentiation by reducing osteoclastogenic cytokines and increasing anti-osteoclastogenic cytokines (59). Recently, it was demonstrated that osteocytes play an important role in inflammatory bone resorption mediated by inflammatory cytokines (108). However, BPs were reported to increase the expression of IL-6 and subsequently trigger elevated RANKL expression in osteocyte-Like MLO-Y4 cells, which potentiates osteoclast formation (109) (**Figure 3**). Thus, it is necessary to further explore the relationship between the alterations induced by BPs and the development of MRONJ.

Cytokines and Osteogenesis

Bone remodeling not only requires osteoclasts to eliminate damaged bone tissue but also requires osteogenesis and angiogenesis to form new bone. In a recent study, it was shown that the occurrence of MRONJ was associated with a deficiency in a subset of γδT cells in human peripheral blood. In addition, it was shown that circulating γδT cells played a pivotal role in bone regeneration rather than just inducing an inflammatory response (110). Furthermore, it was demonstrated that γδT cells can stimulate the release of IL-17A to promote bone formation and the healing of fractures (111).

Platelet-derived growth factor-BB (PDGF-BB), secreted by preosteoclasts, was reported to promote angiogenesis and osteogenesis (63). Gao, et al. reported that zoledronate can inhibit the secretion of PDGF-BB from preosteoclasts, and decreased PDGF-BB suppressed the angiogenic function of endothelial progenitor cells and osteoblastic differentiation of mesenchymal stem cells (112). Moreover, it was found that the number of preosteoclasts and PDGF-BB secretions were significantly reduced in BPs-treated rats coupled with decreased numbers of microvessels and osteoblasts in the early stage of bone healing. Recombinant PDGF-BB rescued the proliferative, migration, and osteogenic functions of impaired mandible-derived bone mesenchymal stem cells in MRONJ-like rats. Finally, PDGF-BB has been confirmed to exhibit therapeutic effects on MRONJ-like rats (113). Together, these results illustrated the important role of PDGF-BB in the development of MRONJ (**Figure 3**).

Experimental data showed that a low dose of BPs significantly increased the expression of genes essential for osteogenic differentiation possibly by upregulating the TGF-β1 production.

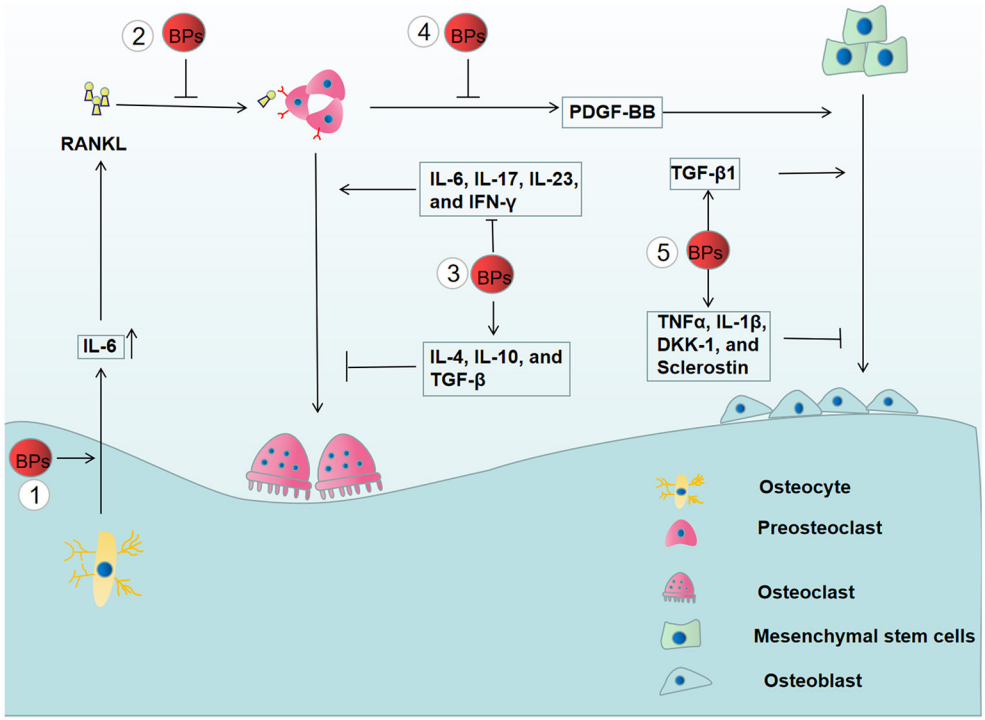

FIGURE 3 | The effects of BPs on bone remodeling. (1) The increased secretion of IL-6 from BPs-treated osteocyte enhances the expression of RANKL, thereby, promoting the osteoclast differentiation. (2) However, BPs inhibit osteoclast differentiation by suppressing the RANKL/OPG/RANK signaling pathway. (3) BPs have an inhibitory effect on the osteoclast differentiation by suppressing the osteoclastogenic cytokines (IL-6, IL-17, IL-23, and IFN-γ) and increasing anti-osteoclastogenic cytokines (IL-4, IL-10, and TGF-β). (4) Osteogenic differentiation is inhibited by the reduced release of PDGF-BB from preosteoclasts. (5) Moreover, BPs affect osteoblastogenesis by altering the release of the pro-inflammatory mediators, including TNFα, IL-1β, DKK-1, sclerostin, and TGF-β1.

These findings provided a new view on the development of MRONJ (114). However, Giannasi et al. found that low doses of BPs could inhibit osteoblastogenesis by enhancing the release of the pro-inflammatory mediators, including TNFα, IL-1β, DKK-1, and Sclerostin (115) (**Figure 3**). Therefore, the role of BPs-mediated osteogenesis in the development of MRONJ is yet not completely understood.

CONCLUSIONS AND FUTURE DIRECTIONS

In this review, we focused on the role of the immune response in the pathogenesis of MRONJ. Treatment with BPs disturbs mucosal immune homeostasis, thereby leading to prolonged oral inflammation and delayed tissue repair. In addition, BPs have an inhibitory effect on the process of bone remodeling in an osteoimmune manner. These results suggested that altered immunity plays an important role in the development of MRONJ. Unfortunately, there are few reports on synergistic or antagonistic effects among various immune cells on the pathogenesis of MRONJ. To better understand the role of the immune response in the development of MRONJ, we should pay more attention to the immune regulation network and the interaction between immune cells and necrosis. Furthermore, current experimental research is predominantly from *ex vivo* studies or animal models and there is a lack of relevant reports on the clinical treatment of MRONJ by immune intervention. It is well-known that there is no significantly effective therapy to cure MRONJ. Recently, there are increasing reports about the application of mucosal vaccination to treat several diseases, such as AIDs (116) and Pertussis (117). Further investigations are required to focus on the treatment of MRONJ using immunotherapy strategies. In addition, it is of great significance to seek excellent biomarkers for early diagnosis and prevention of MRONJ. Several reports have identified that some cytokines are associated with the development of MRONJ. However, the clinical effectiveness and sensitivity of these biomarkers are required to be explored in future studies.

AUTHOR CONTRIBUTIONS

WZ, LG, WR, JZ, ShaoL, and ShasL collected the data. WZ and LG wrote the paper and drew the pictures. CJ, SY, and KZ reviewed and edited the paper. 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.2021.606043/full#supplementary-material

REFERENCES

1. Mücke T, Krestan CR, Mitchell DA, Kirschke JS, Wutzl A. Bisphosphonate and medication-related osteonecrosis of the jaw: a review. *Semin Musculoskelet Radiol.* (2016) 20:305–14. doi: 10.1055/s-0036-1592367
2. Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghaloo T, Mehrotra B, et al. American association of oral and maxillofacial surgeons position paper on medication-related osteonecrosis of the jaw-2014 update. *J Oral Maxillofac Surg.* (2015) 73:1879. doi: 10.1016/j.joms.2014.04.031
3. Campisi G, Mauceri R, Bertoldo F, Bettini G, Biasotto M, Colella G, et al. Medication-Related Osteonecrosis of Jaws (MRONJ) prevention and diagnosis: Italian consensus update 2020. *Int J Environ Res Public Health.* (2020) 17:5998. doi: 10.3390/ijerph17165998
4. Schiodt M, Otto S, Fedele S, Bedogni A, Nicolatou-Galitis O, Guggenberger R, et al. Workshop of European task force on medication-related osteonecrosis of the jaw-current challenges. *Oral Dis.* (2019) 25:1815–21. doi: 10.1111/odi.13160
5. Aljohani S, Fliefel R, Ihbe J, Kühnisch J, Ehrenfeld M, Otto SJJoc-m-fsopotEAfC-M-FS. What is the effect of anti-resorptive drugs (ARDs) on the development of medication-related osteonecrosis of the jaw (MRONJ) in osteoporosis patients: a systematic review. *J Craniomaxillofac Surg.* (2017) 45:1493–502. doi: 10.1016/j.jcms.2017.05.028
6. Messer JG, Calle JLM, Jiron JM, Castillo EJ, Van Poznak C, Bhattacharyya N, et al. Zoledronic acid increases the prevalence of medication-related osteonecrosis of the jaw in a dose dependent manner in rice rats (Oryzomys palustris) with localized periodontitis. *Bone.* (2018) 108:79–88. doi: 10.1016/j.bone.2017.12.025
7. McGowan K, Ivanovski S, Acton C. Osteonecrosis of the jaws: a 14-year retrospective survey of hospital admissions. *Aust Dent J.* (2018) 63:202–7. doi: 10.1111/adj.12603
8. Giovannacci I, Meleti M, Manfredi M, Mortellaro C, Greco Lucchina A, Bonanini M, et al. Medication-related osteonecrosis of the jaw around dental implants: implant surgery-triggered or implant presence-triggered osteonecrosis? *J Craniofac Surg.* (2016) 27:697–701. doi: 10.1097/scs.0000000000002564
9. Soutome S, Hayashida S, Funahara M, Sakamoto Y, Kojima Y, Yanamoto S, et al. Factors affecting development of medication-related osteonecrosis of the jaw in cancer patients receiving high-dose bisphosphonate or denosumab therapy: Is tooth extraction a risk factor? *PLoS ONE.* (2018) 13:e0201343. doi: 10.1371/journal.pone.0201343
10. Lai T, Wang T, Liu C, Chao T, Chen T, Hu YJR, et al. Risk factors for osteonecrosis of the jaw in oral cancer patients after surgery and eventual adjuvant treatment: the potential role of chemotherapy. *Radiother Oncol.* (2017) 123:406–11. doi: 10.1016/j.radonc.2017.05.001
11. Kim T, Kim S, Song M, Lee C, Yagita H, Williams D, et al. Removal of pre-existing periodontal inflammatory condition before tooth extraction ameliorates medication-related osteonecrosis of the jaw-like lesion in mice. *Am J Pathol.* (2018) 188:2318–27. doi: 10.1016/j.ajpath.2018.06.019
12. van Cann T, Loyson T, Verbiest A, Clement PM, Bechter O, Willems L, et al. Incidence of medication-related osteonecrosis of the jaw in patients treated with both bone resorption inhibitors and vascular endothelial growth factor receptor tyrosine kinase inhibitors. *Support Care Cancer.* (2018) 26:869–78. doi: 10.1007/s00520-017-3903-5
13. Chang J, Hakam A, McCauley L. Current understanding of the pathophysiology of osteonecrosis of the jaw. *Curr Osteoporos Rep.* (2018) 16:584–95. doi: 10.1007/s11914-018-0474-4
14. Kalyan S, Wang J, Quabius E, Huck J, Wiltfang J, Baines J, et al. Systemic immunity shapes the oral microbiome and susceptibility to bisphosphonate-associated osteonecrosis of the jaw. *J Transl Med.* (2015) 13:212. doi: 10.1186/s12967-015-0568-z
15. Ruiqing W, Dunfang Z, Eric T, Qianming C, Wanjun C. The mucosal immune system in the oral cavity-an orchestra of T cell diversity. *Int J Oral Sci.* (2014) 6:125–32. doi: 10.1038/ijos.2014.48
16. Nochi T, Kiyono H. Innate immunity in the mucosal immune system. *Curr Pharm Des.* (2006) 12:4203–13. doi: 10.2174/138161206778743457
17. Isolauri E, Sütas Y, Kankaanpää P, Arvilommi H, Salminen S. Probiotics: effects on immunity. *Am J Clin Nutr.* (2001) 73:444S–50S. doi: 10.1093/ajcn/73.2.444s
18. Steevels T, Meyaard L. Immune inhibitory receptors: essential regulators of phagocyte function. *Eur J Immunol.* (2011) 41:575–87. doi: 10.1002/eji.201041179
19. Moutsopoulos N, Konkel J. Tissue-specific immunity at the oral mucosal barrier. *Trends Immunol.* (2018) 39:276–87. doi: 10.1016/j.it.2017.08.005
20. Arweiler N, Netuschil L. The oral microbiota. *Adv Exp Med Biol.* (2016) 902:45–60. doi: 10.1007/978-3-319-31248-4_4
21. Gao L, Xu T, Huang G, Jiang S, Gu Y, Chen F. Oral microbiomes: more and more importance in oral cavity and whole body. *Protein Cell.* (2018) 9:488–500. doi: 10.1007/s13238-018-0548-1
22. Pushalkar S, Li X, Kurago Z, Ramanathapuram L, Matsumura S, Fleisher K, et al. Oral microbiota and host innate immune response in bisphosphonate-related osteonecrosis of the jaw. *Int J Oral Sci.* (2014) 6:219–26. doi: 10.1038/ijos.2014.46
23. Pickard J, Zeng M, Caruso R, Núñez G. Gut microbiota: role in pathogen colonization, immune responses, and inflammatory disease. *Immunol Rev.* (2017) 279:70–89. doi: 10.1111/imr.12567
24. Ermer M, Kottmann S, Otten J, Wittmer A, Poxleitner P, Pelz KJJoo, et al. *In Vitro* investigation of the antimicrobial effect of three bisphosphonates against different bacterial strains. *J Oral Maxillofac Surg.* (2018) 76:553–60. doi: 10.1016/j.joms.2017.08.019
25. Williams DW, Vuong HE, Kim S, Lenon A, Ho K, Hsiao EY, et al. Indigenous microbiota protects against inflammation-induced osteonecrosis. *J Dent Res.* (2020) 99:676–84. doi: 10.1177/0022034520908594
26. Zadik Y. Clarithromycin as the empiric antibiotic therapy for medication-related osteonecrosis of the jaw in multiple myeloma patients. *Oral Oncol.* (2018) 84:104–5. doi: 10.1016/j.oraloncology.2018.07.016
27. Scully C. Oral ulceration: a new and unusual complication. *Br Dent J.* (2002) 192(3):139-40. doi: 10.1038/sj.bdj.4801315
28. Lee R, Sohn S, Shin K, Kang M, Park N, Kim R. Bisphosphonate inhibits the expression of cyclin A2 at the transcriptional level in normal human oral keratinocytes. *Int J Mol Med.* (2017) 40:623–30. doi: 10.3892/ijmm.2017.3066
29. McLeod N, Moutasim K, Brennan P, Thomas G, Jenei, V. *In vitro* effect of bisphosphonates on oral keratinocytes and fibroblasts. *J Oral Maxillofac Surg.* (2014) 72:503–9. doi: 10.1016/j.joms.2013.08.007
30. Kim RH, Lee RS, Williams D, Bae S, Woo J, Lieberman M, et al. Bisphosphonates induce senescence in normal human oral keratinocytes. *J Dent Res.* (2011) 90:810–6. doi: 10.1177/0022034511402995
31. Pabst A, Ziebart T, Koch F, Taylor K, Al-Nawas B, Walter C. The influence of bisphosphonates on viability, migration, and apoptosis of human oral keratinocytes–*in vitro* study. *Clin Oral Investig.* (2012) 16:87–93. doi: 10.1007/s00784-010-0507-6
32. Ziebart T, Halling F, Heymann P, Neff A, Blatt S, Jung J, et al. Impact of soft tissue pathophysiology in the development and maintenance of Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ). *Dent J.* (2016) 4:36. doi: 10.3390/dj4040036

33. Mehta M, Ahmed S, Dryden G. Immunopathophysiology of inflammatory bowel disease: how genetics link barrier dysfunction and innate immunity to inflammation. *Innate Immun.* (2017) 23:497–505. doi: 10.1177/1753425917722206
34. Medzhitov R. Origin and physiological roles of inflammation. *Nature.* (2008) 454:428–35. doi: 10.1038/nature07201
35. Shuster A, Reiser V, Trejo L, Ianculovici C, Kleinman S, Kaplan I, et al. Comparison of the histopathological characteristics of osteomyelitis, medication-related osteonecrosis of the jaw, and osteoradionecrosis. *Int J Oral Maxillofac Surg.* (2019) 48:17–22. doi: 10.1016/j.ijom.2018.07.002
36. Sedghizadeh PP, Kumar SKS, Gorur A, Schaudinn C, Shuler CF, Costerton JW. Microbial biofilms in osteomyelitis of the jaw and osteonecrosis of the jaw secondary to bisphosphonate therapy. *J Am Dent Assoc.* (2009) 140:1259-65. doi: 10.14219/jada.archive.2009.0049
37. Heyman O, Koren N, Mizraji G, Capucha T, Wald S, Nassar M, et al. Impaired differentiation of langerhans cells in the murine oral epithelium adjacent to titanium dental implants. *Front Immunol.* (2018) 9:1712. doi: 10.3389/fimmu.2018.01712
38. Jie Z, Yang J, Gu M, Wang H, Xie X, Li Y, et al. NIK signaling axis regulates dendritic cell function in intestinal immunity and homeostasis. *Nat Immunol.* (2018) 19:1224–35. doi: 10.1038/s41590-018-0206-z
39. Luan Y, Zhang L, Zhu F, Dong N, Lu J, Yao YM. Effect of TIPE1 on Immune Function of Dendritic Cells and Its Signaling Pathway in Septic Mice. *J Infect Dis.* (2019) 220:699–709. doi: 10.1093/infdis/jiz158
40. Coughlin M, Bellini W, Rota PA. Contribution of dendritic cells to measles virus induced immunosuppression. *Rev Med Virol.* (2013) 23:126–38. doi: 10.1002/rmv.1735
41. Bringmann A, Schmidt S, Weck M, Brauer K, von Schwarzenberg K, Werth D, et al. Zoledronic acid inhibits the function of Toll-like receptor 4 ligand activated monocyte-derived dendritic cells. *Leukemia.* (2007) 21:732–8. doi: 10.1038/sj.leu.2404556
42. Orsini G, Failli A, Legitimo A, Adinolfi B, Romanini A, Consolini R, et al. Zoledronic acid modulates maturation of human monocyte-derived dendritic cells. *Exp Biol Med.* (2011) 236:1420–6. doi: 10.1258/ebm.2011.011168
43. Elsayed R, Kurago Z, Cutler C, Arce R, Gerber J, Celis E, et al. Role of dendritic cell-mediated immune response in oral homeostasis: a new mechanism of osteonecrosis of the jaw. *FASEB J.* (2020) 34:2595–608. doi: 10.1096/fj.201901819RR
44. Wynn TA, Vannella KM. Macrophages in tissue repair, regeneration, and fibrosis. *Immunity.* (2016) 44:450–62. doi: 10.1016/j.immuni.2016.02.015
45. Bain C, Mowat AM. Macrophages in intestinal homeostasis and inflammation. *Immunol Rev.* (2014) 260:102–17. doi: 10.1111/imr.12192
46. Hoefert S, Schmitz I, Weichert F, Gaspar M, Eufinger H. Macrophages and bisphosphonate-related osteonecrosis of the jaw (BRONJ): evidence of local immunosuppression of macrophages in contrast to other infectious jaw diseases. *Clin Oral Investig.* (2015) 19:497–508. doi: 10.1007/s00784-014-1273-7
47. Davies LC, Taylor PR. Tissue-resident macrophages: then and now. *Immunology.* (2015) 144:541–8. doi: 10.1111/imm.12451
48. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat Rev Immunol.* (2014) 14:392–404. doi: 10.1038/nri3671
49. Pan F, Tang W, Zhou Z, Gilkeson G, Lang R, Jiang WJL. Intestinal macrophages in mucosal immunity and their role in systemic lupus erythematosus disease. *Lupus.* (2018) 27:1898–902. doi: 10.1177/0961203318797417
50. Hoefert S, Sade Hoefert C, Munz A, Northoff H, Yuan A, Reichenmiller K, et al. Altered macrophagic THP-1 cell phagocytosis and migration in bisphosphonate-related osteonecrosis of the jaw (BRONJ). *Clin Oral Investig.* (2016) 20:1043–54. doi: 10.1007/s00784-015-1584-3
51. Hayano H, Kuroshima S, Sasaki M, Tamaki S, Inoue M, Ishisaki A, et al. Distinct immunopathology in the early stages between different antiresorptives-related osteonecrosis of the jaw-like lesions in mice. *Bone.* (2020) 135:115308. doi: 10.1016/j.bone.2020.115308
52. Hoefert S, Hoefert CS, Albert M, Munz A, Grimm M, Northoff H, et al. Zoledronate but not denosumab suppresses macrophagic differentiation of THP-1 cells. An aetiologic model of bisphosphonate-related osteonecrosis of the jaw (BRONJ). *Clin Oral Investig.* (2015) 19:1307–18. doi: 10.1007/s00784-014-1358-3
53. Ruytinx P, Proost P, Van Damme J, Struyf S. Chemokine-Induced Macrophage Polarization in Inflammatory Conditions. *Front Immunol.* (2018) 9:1930. doi: 10.3389/fimmu.2018.01930
54. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili S, Mardani F, et al. Macrophage plasticity, polarization, and function in health and disease. *J Cell Physiol.* (2018) 233:6425–40. doi: 10.1002/jcp.26429
55. Funes S, Rios M, Escobar-Vera J, Kalergis AM. Implications of macrophage polarization in autoimmunity. *Immunology.* (2018) 154:186–95. doi: 10.1111/imm.12910
56. Paschalidi P, Gkouveris I, Soundia A, Kalfarentzos E, Vardas E, Georgaki M, et al. The role of M1 and M2 macrophage polarization in progression of medication-related osteonecrosis of the jaw. *Clin Oral Investig.* (2020). doi: 10.1007/s00784-020-03602-z. [Epub ahead of print].
57. Zhu W, Xu R, Du J, Fu Y, Li S, Zhang P, et al. Zoledronic acid promotes TLR-4-mediated M1 macrophage polarization in bisphosphonate-related osteonecrosis of the jaw. *FASEB J.* (2019) 33:5208–19. doi: 10.1096/fj.201801791RR
58. Zhang Q, Yu W, Lee S, Xu Q, Naji A, Le, AD. Bisphosphonate induces osteonecrosis of the jaw in diabetic mice via NLRP3/Caspase-1-Dependent IL-1β Mechanism. *J Bone Miner Res.* (2015) 30:2300–12. doi: 10.1002/jbmr.2577
59. Talaat R, Sidek A, Mosalem A, Kholief AJI. Effect of bisphosphonates treatment on cytokine imbalance between TH17 and Treg in osteoporosis. *Inflammopharmacology.* (2015) 23:119–25. doi: 10.1007/s10787-015-0233-4
60. Pelegrin P, Surprenant A. Dynamics of macrophage polarization reveal new mechanism to inhibit IL-1beta release through pyrophosphates. *EMBO J.* (2009) 28:2114–27. doi: 10.1038/emboj.2009.163
61. Zhang Q, Atsuta I, Liu S, Chen C, Shi S, Shi S, et al. IL-17-mediated M1/M2 macrophage alteration contributes to pathogenesis of bisphosphonate-related osteonecrosis of the jaws. *Clin Cancer Res.* (2013) 19:3176–88. doi: 10.1158/1078-0432.CCR-13-0042
62. Sol K, Drake W, Cindy L, Terresa K, Atsushi A, Songtao S , et al. IL-36 induces bisphosphonate-related osteonecrosis of the jaw-like lesions in mice by inhibiting TGF-β-mediated collagen expression. *J Bone Miner Res.* (2017) 32:309–18. doi: 10.1002/jbmr.2985
63. Tao X, YongJun L, FanQi K, Bin L, ShuJie Z, Jian C, et al. GIT1 is critical for formation of the CD31Emcn vessel subtype in coupling osteogenesis with angiogenesis via modulating preosteoclasts secretion of PDGF-BB. *Bone.* (2019) 122:218–30. doi: 10.1016/j.bone.2019.03.006
64. Alexandru M, Hani M, Kazuaki N, Tomomi K, Kenji E, Jae-Young K, et al. Possible pathogenic engagement of soluble Semaphorin 4D produced by γδT cells in medication-related osteonecrosis of the jaw (MRONJ). *Biochem Biophys Res Commun.* (2016) 480:42–7. doi: 10.1016/j.bbrc.2016.10.012
65. Kaneko J, Okinaga T, Hikiji H, Ariyoshi W, Yoshiga D, Habu M, et al. Zoledronic acid exacerbates inflammation through M1 macrophage polarization. *Inflamm Regen.* (2018) 38:16. doi: 10.1186/s41232-018-0074-9
66. Dolasia K, Bisht MK, Pradhan G, Udgata A, Mukhopadhyay S. TLRs/NLRs: Shaping the landscape of host immunity. *Int Rev Immunol.* (2018) 37:3–19. doi: 10.1080/08830185.2017.1397656
67. Schamel WW, Alarcon B, Minguet S. The TCR is an allosterically regulated macromolecular machinery changing its conformation while working. *Immunol Rev.* (2019) 291:8–25. doi: 10.1111/imr.12788
68. Muro R, Takayanagi H, Nitta T. T cell receptor signaling for γδT cell development. *Inflamm Regen.* (2019) 39:6. doi: 10.1186/s41232-019-0095-z
69. Hovav A, Wilharm A, Barel O, Prinz I. Development and Function of γδT Cells in the Oral Mucosa. *J Dent Res.* (2020) 99:498–505. doi: 10.1177/0022034520908839
70. Clézardin P, Massaia M. Nitrogen-containing bisphosphonates and cancer immunotherapy. *Curr Pharm Des.* (2010) 16:2014–3007. doi: 10.2174/138161210793563545

71. Kalyan S, Quabius ES, Wiltfang J, Mönig H, Kabelitz D. Can peripheral blood γδ T cells predict osteonecrosis of the jaw? An immunological perspective on the adverse drug effects of aminobisphosphonate therapy. *J Bone Miner Res.* (2013) 28:728–35. doi: 10.1002/jbmr.1769
72. Roelofs AJ, Jauhiainen M, Mönkkönen H, Rogers MJ, Mönkkönen J, Thompson K. Peripheral blood monocytes are responsible for gammadelta T cell activation induced by zoledronic acid through accumulation of IPP/DMAPP. *Br J Haematol.* (2009) 144:245–50. doi: 10.1111/j.1365-2141.2008.07435.x
73. Park S, Kanayama K, Kaur K, Tseng H, Banankhah S, Quje D, et al. Osteonecrosis of the Jaw Developed in Mice: disease variants regulated by γδT cells in oral mucosal barrier immunity. *J Biol Chem.* (2015) 290:17349–66. doi: 10.1074/jbc.M115.652305
74. Green DR, Droin N, Pinkoski M. Activation-induced cell death in T cells. *Immunol Rev.* (2003) 193:70–81. doi: 10.1034/j.1600-065x.2003.00051.x
75. Kalyan S, Chandrasekaran V, Quabius ES, Lindhorst TK, Kabelitz D. Neutrophil uptake of nitrogen-bisphosphonates leads to the suppression of human peripheral blood γδ T cells. *Cell Mol Life Sci.* (2014) 71:2335–46. doi: 10.1007/s00018-013-1495-x
76. Pino-Tamayo PA, Puerta-Arias JD, Lopera D, Urán-Jiménez ME, González Á. Depletion of Neutrophils Exacerbates the Early Inflammatory Immune Response in Lungs of Mice Infected with Paracoccidioides brasiliensis. *Mediators Inflamm.* (2016) 2016:3183285. doi: 10.1155/2016/3183285
77. Zeng M, Miralda I, Armstrong C, Uriarte S, Bagaitkar J. The roles of NADPH oxidase in modulating neutrophil effector responses. *Mol Oral Microbiol.* (2019) 34:27–38. doi: 10.1111/omi.12252
78. Hagelauer N, Pabst AM, Ziebart T, Ulbrich H, Walter C. *In vitro* effects of bisphosphonates on chemotaxis, phagocytosis, and oxidative burst of neutrophil granulocytes. *Clin Oral Investig.* (2015) 19:139–48. doi: 10.1007/s00784-014-1219-0
79. de Barros Silva PG, de Oliveira CC, Brizeno LA, Wong DV, Lima Júnior RC, Gonçalves RP, et al. Immune cellular profile of bisphosphonate-related osteonecrosis of the jaw. *Oral Dis.* (2016) 22:649–57. doi: 10.1111/odi.12513
80. Kuiper JWP, Forster C, Sun C, Peel S, Glogauer M. Zoledronate and pamidronate depress neutrophil functions and survival in mice. *Br J Pharmacol.* (2012) 165:532–9. doi: 10.1111/j.1476-5381.2011.01592.x
81. Favot CL, Forster C, Glogauer M. The effect of bisphosphonate therapy on neutrophil function: a potential biomarker. *Int J Oral Maxillofac Surg.* (2013) 42:619–26. doi: 10.1016/j.ijom.2012.12.011
82. Scheper MA, Badros A, Chaisuparat R, Cullen KJ, Meiller TF. Effect of zoledronic acid on oral fibroblasts and epithelial cells: a potential mechanism of bisphosphonate-associated osteonecrosis. *Br J Haematol.* (2009) 144:667–76. doi: 10.1111/j.1365-2141.2008.07504.x
83. Saito T, Izumi K, Shiomi A, Uenoyama A, Ohnuki H, Kato H, et al. Zoledronic acid impairs re-epithelialization through downregulation of integrin alpha v beta 6 and transforming growth factor beta signalling in a three-dimensional *in vitro* wound healing model. *Int J Oral Maxillofac Surg.* (2014) 43:373–80. doi: 10.1016/j.ijom.2013.06.016
84. Yuan A, Munz A, Reinert S, Hoefert SJJoc-m-fsopotEAfC-M-FS. Gingival fibroblasts and medication-related osteonecrosis of the jaw: Results by real-time and wound healing *in vitro* assays. *J Craniomaxillofac Surg.* (2019) 47:1464–74. doi: 10.1016/j.jcms.2019.06.004
85. Tamaki S, Kuroshima S, Hayano H, Nakajima K, Kakehashi H, Ishisaki A, et al. Dynamic polarization shifting from M1 to M2 macrophages in reduced osteonecrosis of the jaw-like lesions by cessation of anti-RANKL antibody in mice. *Bone.* (2020)141:115560. doi: 10.1016/j.bone.2020.115560
86. Wang Q, Liu J, Guo T, Liu D, Pan J. Epidermal Growth Factor Reverses the Inhibitory Effects of the Bisphosphonate, Zoledronic Acid, on Human Oral Keratinocytes and Human Vascular Endothelial Cells *in Vitro via* the Epidermal Growth Factor Receptor (EGFR)/Akt/Phosphoinositide 3-Kinase (PI3K) Signaling Pathway. *Med Sci Monit.* (2019) 25:700–10. doi: 10.12659/msm.911579
87. Mawardi H, Giro G, Kajiya M, Ohta K, Almazrooa S, Alshwaimi E, et al. A role of oral bacteria in bisphosphonate-induced osteonecrosis of the jaw. *J Dent Res.* (2011) 90:1339–45. doi: 10.1177/0022034511420430
88. Tamari T, Elimelech R, Cohen G, Cohen T, Doppelt O, Eskander-Hashoul L, et al. Endothelial Progenitor Cells inhibit jaw osteonecrosis in a rat model: a major adverse effect of bisphosphonate therapy. *Sci Rep.* (2019) 9:18896. doi: 10.1038/s41598-019-55383-5
89. Yamashita J, Koi K, Yang D-Y, McCauley LK. Effect of zoledronate on oral wound healing in rats. *Clin Cancer Res.* (2011) 17:1405–14. doi: 10.1158/1078-0432.CCR-10-1614
90. Rao SH, Harini B, Shadamarshan RPK, Balagangadharan K, Selvamurugan N. Natural and synthetic polymers/bioceramics/bioactive compounds-mediated cell signalling in bone tissue engineering. *Int J Biol Macromol.* (2018) 110:88–96. doi: 10.1016/j.ijbiomac.2017.09.029
91. Scholtysek C, Krönke G, Schett G. Inflammation-associated changes in bone homeostasis. *Inflamm Allergy Drug Targets.* (2012) 11:188–95. doi: 10.2174/187152812800392706
92. Kinane DF, Stathopoulou PG, Papapanou PN. Periodontal diseases. *Nat Rev Dis Primers.* (2017) 3:17038. doi: 10.1038/nrdp.2017.38
93. Dar HY, Azam Z, Anupam R, Mondal RK, Srivastava RK. Osteoimmunology: the between bone and immune system. *Front Biosci.* (2018) 23:464–92. doi: 10.2741/4600
94. Terashima A, Takayanagi H. Overview of osteoimmunology. *Calcif Tissue Int.* (2018) 102:503–11. doi: 10.1007/s00223-018-0417-1
95. Sinder BP, Pettit AR, McCauley LK. Macrophages: their emerging roles in bone. *J Bone Miner Res.* (2015) 30:2140–9. doi: 10.1002/jbmr.2735
96. Moriceau G, Ory B, Gobin B, Verrecchia F, Gouin F, Blanchard F, et al. Therapeutic approach of primary bone tumours by bisphosphonates. *Curr Pharm Des.* (2010) 16:2981–7. doi: 10.1007/s00223-017-0372-2
97. Terpos E, Dimopoulos MA. Interaction between the skeletal and immune systems in cancer: mechanisms and clinical implications. *Cancer Immunol Immunother.* (2011) 60:305–17. doi: 10.1007/s00262-011-0974-x
98. Hoefert S, Schmitz I, Tannapfel A, Eufinger H. Importance of microcracks in etiology of bisphosphonate-related osteonecrosis of the jaw: a possible pathogenetic model of symptomatic and non-symptomatic osteonecrosis of the jaw based on scanning electron microscopy findings. *Clin Oral Investig.* (2010) 14:271–84. doi: 10.1007/s00784-009-0300-6
99. Izawa T, Ishimaru N, Moriyama K, Kohashi M, Arakaki R, Hayashi Y. Crosstalk between RANKL and Fas signaling in dendritic cells controls immune tolerance. *Blood.* (2007) 110:242–50. doi: 10.1182/blood-2006-11-059980
100. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat Rev Immunol.* (2007) 7:292–304. doi: 10.1038/nri2062
101. Weiqiao Z, Xing W, Xiaoxia W, Zhiying W. Temporal and spatial expression of osteoprotegerin and receptor activator of nuclear factor -kappaB ligand during mandibular distraction in rats. *J Craniomaxillofac Surg.* (2007) 35:103–11.doi: 10.1016/j.jcms.2006.12.001
102. Di Nisio C, Zizzari V, Zara S, Falconi M, Teti G, Tetè G, et al. RANK/RANKL/OPG signaling pathways in necrotic jaw bone from bisphosphonate-treated subjects. *Eur J Histochem.* (2015) 59:2455. doi: 10.4081/ejh.2015.2455
103. Mercatali L, Ricci M, Scarpi E, Serra P, Fabbri F, Ricci R, et al. RANK/RANK-L/OPG in patients with bone metastases treated with anticancer agents and zoledronic acid: a prospective study. *Int J Mol Sci.* (2013) 14:10683–93. doi: 10.3390/ijms140610683
104. Nakagawa T, Ohta K, Kubozono K, Ishida Y, Naruse T, Takechi M, et al. Zoledronate inhibits receptor activator of nuclear factor kappa-B ligand-induced osteoclast differentiation via suppression of expression of nuclear factor of activated T-cell c1 and carbonic anhydrase 2. *Arch Oral Biol.* (2015) 60:557–65. doi: 10.1016/j.archoralbio.2014.09.012
105. Li G, Tang X, Zhang S, Jin M, Wang M, Deng Z, et al. SIRT7 activates quiescent hair follicle stem cells to ensure hair growth in mice. *EMBO J.* (2020) 39:e104365. doi: 10.15252/embj.201910 4365
106. Huang X, Huang L, Cheng Y, Li F, Zhou Q, Wu C, et al. Zoledronic acid inhibits osteoclast differentiation and function through the regulation of NF-κB and JNK signalling pathways. *Int J Mol Med.* (2019) 44:582–92. doi: 10.3892/ijmm.2019.4207
107. Amarasekara D, Yun H, Kim S, Lee N, Kim H, Rho J. Regulation of osteoclast differentiation by cytokine networks. *Immune Netw.* (2018) 18:e8. doi: 10.4110/in.2018.18.e8

108. Marahleh A, Kitaura H, Ohori F, Kishikawa A, Ogawa S, Shen W, et al. TNF-α directly enhances osteocyte rankl expression and promotes osteoclast formation. *Front Immunol.* (2019) 10:2925. doi: 10.3389/fimmu.2019.02925
109. Kim H, Kim H, Choi Y, Bae M, Hwang D, Shin S, et al. Zoledronate enhances osteocyte-mediated osteoclast differentiation by IL-6/RANKL Axis. *Int J Mol Sci.* (2019) 20:1467. doi: 10.3390/ijms20061467
110. Kalyan S. It May Seem Inflammatory, but Some T Cells Are Innately Healing to the Bone. *J Bone Miner Res.* (2016) 31:1997–2000. doi: 10.1002/jbmr.2875
111. Takehito O, Kazuo O, Tomoki N, Takeshi N, Shohei H, Yoichiro I, et al. IL-17-producing γδ T cells enhance bone regeneration. *Nat Commun.* (2016) 7:10928. doi: 10.1038/ncomms10928
112. Siyong G, Guangsen Z, Lin W, Yujie L, Sien Z, Xiaomei L, et al. Zoledronate suppressed angiogenesis and osteogenesis by inhibiting osteoclasts formation and secretion of PDGF-BB. *PLoS ONE.* (2017) 12:e0179248. doi: 10.1371/journal.pone.0179248
113. Siyong G, Ruibang L, Sihui H, Yujie L, Xiang L, Sien Z, et al. PDGF-BB exhibited therapeutic effects on rat model of bisphosphonate-related osteonecrosis of the jaw by enhancing angiogenesis and osteogenesis. *Bone.* (2019) 114:115117. doi: 10.1016/j.bone.2019.115117
114. Manzano-Moreno F, Ramos-Torrecillas J, Melguizo-Rodríguez L, Illescas-Montes R, Ruiz C, García-Martínez, O. Bisphosphonate modulation of the gene expression of different markers involved in osteoblast physiology: possible implications in bisphosphonate-related osteonecrosis of the jaw. *Int J Med Sci.* (2018) 15:359–67. doi: 10.7150/ijms.22627
115. Giannasi C, Niada S, Farronato D, Lombardi G, Manfredi B, Farronato G, et al. Nitrogen Containing bisphosphonates impair the release of bone homeostasis mediators and matrix production by human primary pre-osteoblasts. *Int J Med Sci.* (2019) 16:23–32. doi: 10.7150/ijms.27470
116. Aldovini A. Mucosal Vaccination for Prevention of HIV Infection and AIDS. *Curr HIV Res.* (2016) 14:247–59. doi: 10.2174/1570162x14999160224103025
117. Solans L, Locht C. The role of mucosal immunity in pertussis. *Front Immunol.* (2018) 9:3068. doi: 10.3389/fimmu.2018.03068

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REVIEW
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Dysbiosis From a Microbial and Host Perspective Relative to Oral Health and Disease

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The significance of microbiology and immunology with regard to caries and periodontal disease gained substantial clinical or research consideration in the mid 1960's. This enhanced emphasis related to several simple but elegant experiments illustrating the relevance of bacteria to oral infections. Since that point, the understanding of oral diseases has become increasingly sophisticated and many of the original hypotheses related to disease causality have either been abandoned or amplified. The COVID pandemic has reminded us of the importance of history relative to infectious diseases and in the words of Churchill "those who fail to learn from history are condemned to repeat it." This review is designed to present an overview of broad general directions of research over the last 60 years in oral microbiology and immunology, reviewing significant contributions, indicating emerging foci of interest, and proposing future directions based on technical advances and new understandings. Our goal is to review this rich history (standard microbiology and immunology) and point to potential directions in the future (omics) that can lead to a better understanding of disease. Over the years, research scientists have moved from a position of downplaying the role of bacteria in oral disease to one implicating bacteria as true pathogens that cause disease. More recently it has been proposed that bacteria form the ecological first line of defense against "foreign" invaders and also serve to train the immune system as an acquired host defensive stimulus. While early immunological research was focused on immunological exposure as a modulator of disease, the "hygiene hypothesis," and now the "old friends hypothesis" suggest that the immune response could be trained by bacteria for long-term health. Advanced "omics" technologies are currently being used to address changes that occur in the host and the microbiome in oral disease. The "omics" methodologies have shaped the detection of quantifiable biomarkers to define human physiology and pathologies. In summary, this review will emphasize the role that commensals and pathobionts play in their interaction with the immune status of the host, with a prediction that current "omic" technologies will allow researchers to better understand disease in the future.

Keywords: oral microbiology, oral immunology, commensal, pathobiont, metaproteomics, metabolomics, periodontitis, caries

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THE ROLE OF MICROORGANISMS IN DISEASE

Introduction

In the context of homeostatic balance between the host and its microbial content, it has been proposed that the commensal microbiota plays a critical role in maintaining health (Lloyd-Price et al., 2016). Disruption of this homeostatic balance, is known as dysbiosis, defined as perturbations in the composition of commensal communities relative to that found in health (Petersen and Round, 2014; Lloyd-Price et al., 2016; Hooks and O'Malley, 2017). Dysbiosis can occur as a result of a change in the microbiota or the host's ability to respond to its microbiota. This delicate balance between homeostasis and dysbiosis is in part now seen as the early training of local and systemic immune regulation (both innate and acquired regulators) (Alm et al., 2002; Rook et al., 2013; Cox et al., 2014). Conceptually, this concept can be illustrated in germ-free animals where a lack of exposure to a typical commensal microbiota leads to an immature/untrained immune system (Falk et al., 1998; Macpherson and Harris, 2004; Round and Mazmanian, 2009; Sommer and Backhed, 2013; Kennedy et al., 2018). In contrast, early exposure and immune training as seen in immune competent humans, has been termed the "old friends" hypothesis, which describes the manner by which the host responds to microbial challenges (Rook, 2010; Cox et al., 2014; Rook et al., 2014). The disturbance of training of the immune system, also seen in humans, has been illustrated by challenging the infant commensal microbiota with antibiotics, which can disrupt this homeostatic balance (Dominguez-Bello et al., 2019). Moreover, antibiotics given to infant mice can change their gut (and likely oral) microbiota, suppress the normal (eubiotic) commensal microbiota, and add weight (obesity) and height to these antibiotic-treated mice (Cox et al., 2014; Lamont et al., 2018).

Further substantiation of this need for training and balance has been shown by removal of intestinal microbial contents from non-antibiotic treated mice followed by transplantation of the intestinal microbiota from antibiotic treated infant mice as compared to non-antibiotic treated mice (Cox et al., 2014; Ellekilde et al., 2014). When challenged, these two transplanted populations were shown to produce distinctive responses in the mice receiving the transplants. The newborn mice receiving the transplants from antibiotic treated mice produce heavier and larger mice as compared to those mice receiving the "normal" non-antibiotic manipulated microbiota (Cox et al., 2014). In experiments by Ellekilde et al. (2014) the ultimate goal was to develop a system designed to circumvent the need for germ free mice in order to document microbiome development and its effect on the host. Colonization of transplanted mice was assessed comparing donors from either lean or obese mice. The transplantation effect, in these experiments, although stable for only 6 weeks, was sufficient to train the immune system and allow for the study of pathogenesis in murine models of disease.

The importance of this "early" commensal gut microbiota in brain development has also been described in both studies with mice and humans (Lu et al., 2018; Lu and Claud, 2019). Proper neurological development appears to be intimately tied to maintenance of a healthy microbiome, and alternations or dysbiosis appear to be linked to schizophrenia, autism spectrum disorders, and hyperactivity disorders (Hsiao et al., 2013; Kong et al., 2019; Lu and Claud, 2019). Gnotobiotic mice humanized with transplanted early fecal microbiota from preterm infants with either good or poor growth, and mouse brain, liver, fecal, and serum samples were obtained to analyze histology, protein, fatty acid, and RNA expression levels in these transplanted mice (Lu et al., 2018). Mice that were colonized by poor-growth microbes showed decreased levels of markers of early development in the brain, and delayed oligodendrocyte development and myelination, indicating a delay in neuronal development. Furthermore, in the poor-growth mice, neurotransmitter levels were altered and animals developed neuroinflammation. There was a subsequent change in the short chain fatty acids from the gut microbiota. This study demonstrated the profound effect the colonizing microbiome had on early brain development, validating many of the theories and prior data linking the gut-brain axis in neuronal development (Lu et al., 2018).

Are Microbes Alone Responsible for Health or Disease?

This "old friends" mechanism coupled with the important work of Casadevall and Pirofski clearly point out that it is inadequate and misleading to define disease based solely the virulence capability of specific microbes and the so-called "pathogenic microbiota" (Casadevall and Pirofski, 2015, 2018). Concomitantly, health should be defined in terms of the influence of the commensal microbiota ("old friends") on its host response capabilities. Therefore, the host as well as the microbiota should be included when assessing health or disease (Dominguez-Bello et al., 2019). From the perspective of disease, this is best explained in the Damage/Response Framework that demands that we define disease in the context of the host (Casadevall and Pirofski, 2003; Pirofski and Casadevall, 2008). Thus, typically harmless bacteria can become opportunistic or pathogenic, and are capable of inducing disease when introduced into an organ system that is not its normal ecological niche especially in immunologically compromised individuals (Casadevall and Pirofski, 1999). From the perspective of health, one should consider the importance of the commensal microbiota and how its absence or alteration can undermine immune surveillance and influence growth, development, and resistance to disease (Dominguez-Bello et al., 2019). As mentioned in the Damage/Response Framework a defective immune system can have severe clinical implications in disease. This was illustrated initially in the case of HIV/AIDS (Casadevall and Pirofski, 1999). In the Framework proposed in this conceptualization of disease, microbes such as the opportunistic pathogen *Cryptococcus* sp., or commensals like *Candida* sp. or *Staphylococcus* sp., that are ordinarily controlled by immune competence, now become the cause of morbidity and mortality. Thus, in the Damage/Response Framework generally "harmless" bacteria, fungi, and viruses now run rampant and cause fulminating infections that cannot be controlled at either the local of systemic level (Casadevall and Pirofski, 1999).

How Does the Commensal Microbiota Evolve?

Higher order organisms acquire their microbiomes from their immediate environment upon birth initially through oral feeding. Mammals are known to be colonized initially by organisms present in the birth canal and passively from primary caregivers during rearing (Berkowitz and Jones, 1985; Lamell et al., 2000; Mueller et al., 2015). Barring any disruption from outside influences (i.e., antibiotics while *in utero* or early in the colonization process) or imbalances in the maternal microbiome, offspring will acquire their healthy commensal microbiome early in their development (Cho and Blaser, 2012). Commensals, our "old friends," play an important role in homeostasis, disease control through competition (i.e., for nutrients) and exclusion (i.e., inhibitory compound production) of pathogens, maintaining health at local sites through adequate colonization rates, metabolic activity, and immune training (Relman, 2012; Abt and Pamer, 2014). The importance of the commensal microbiota in the protection from potentially pathogenic species is well-illustrated in the case of commensal *Neisseria* species of the oro-nasopharynx (Dorey et al., 2019). *Neisseria*, which are regarded as benign common colonizers of the mouth and nasal cavities are generally able to manage pathogenic or pathobiotic *Neisseria* species. For instance, indigenous *Neisseria* can kill the potential pathogenic *Neisseria gonorrhoeae,* or likely outcompete the pathobiont *Neisseria meningitidis* (Pandey et al., 2018; Dorey et al., 2019; Kim et al., 2019).

It has now become clear that there is a selective process that determines the colonizing order and distribution of microorganisms that gather on epithelial and hard surfaces forming the microbiome of humans and other mammals (Shafquat et al., 2014; Lloyd-Price et al., 2016). Previous theories suggested that microorganisms were ubiquitous, completely surrounding and inhabiting us based on selection preferences that were poorly defined. Concisely put, the prevailing theory was that "everything is everywhere" (O'Malley, 2007). Overtime this concept has evolved into a more precise definition of ecological selection (Costello et al., 2012; Foster et al., 2017). Currently, the local environment still forms a key element in the selection process. However, contemporary concepts now indicate that both the niche microbiota and the host response to that microbiota serve as a filter for microbial selection and the successional development of specific habitats (Human Microbiome Project, 2012; Foster et al., 2017).

STUDIES POINTING TO THE IMPORTANCE OF MICROBIAL AND HOST INVOLVEMENT IN INFECTIOUS DISEASES

Interest in the Prominence of Dysbiotic Microbial Communities

In a natural state, most niches are filled by a climax community that has unique physiological and/or metabolic demands that therefore can restrict invasion or colonization of non-niche or transient species. However, successful disruption of this community ("patch or domain") will permit shifts in the established inhabitants that can lead to dysbiotic behavior and potentially pathogenic communities (Relman, 2012). Microbial species that are original occupants of a specific niche are functionally fit for that ecological niche (Polechová and Storch, 2019). Interconnecting food chains allow for a multitude of physiological functions that permit microbial diversity in a "climax community" (Jorth et al., 2014). Dispersal, local diversification, environmental selection, or, ecological drift can allow for subtle or not so subtle shifts in the climax community (Costello et al., 2012).

Chemical and/or Physical Causes of Ecological Disruption

To illustrate this more specifically either chemical or physical disruptions can lead to microbial community imbalances or dysbiosis. Chemically induced environmental dysbiosis can be seen in the overuse of antibiotics, which creates an ecological catastrophe, particular within the gastrointestinal (GI) microbiota (Relman, 2012; Dominguez-Bello et al., 2019; Cullen et al., 2020).

In the past, Clindamycin had the highest association with GI disturbance and serious consequences among antibiotics studied (Sullivan et al., 2001; Brown et al., 2013). In addition, older patients appeared to be more susceptible to the effect of Clindamycin in its ability to disrupt intestinal equilibrium (Loo et al., 2011). The overall consequence was the overgrowth of *Clostridium difficile*, and suppression of microbes that could counter its effect and thus disrupt homeostasis. In a mouse model a single dose of clindamycin was able to render the animals susceptible to *C. difficile*-induced colitis (Buffie et al., 2012). This is just one example of chemically induced environmentally initiated dysbiosis resulting from the misuse of antibiotics, which creates an ecological catastrophe.

Recent work addressing the impact of orally administered prophylactic antibiotics on the gut microbiota of hematology patients was observed (Willmann et al., 2019). Patients were immunocompromised due to their malignancies and were administered either ciprofloxacin or co-trimoxazole daily depending on the study site. Baseline laboratory tests were performed to assess liver function and markers of infection. Study participants gave stool samples prior to antibiotic treatment, days 1 and 3 post-initiation of treatment, and in the final antibiotic dosage period, which had a 6-day median time period. The samples were evaluated by shotgun sequencing, whereby in addition to speciation, the resistome and plasmidome could be analyzed. In both groups there was an observed decline in the Shannon diversity at a phylum level over the course of the treatment, which showed specific microbial species depending on the antibiotic. Antibiotic resistance genes increased over the course of the study, but the specific genes were dependent on the antibiotic. Interestingly, the patient's laboratory findings also correlated with alternations in the microbiota, pointing to the role the host has in shaping the gut microbiome.

Another example of the importance of the normal commensal microbiota and a consequence of its disruption can be shown by the homeostatic microbial imbalance and immune disruption caused by overuse of antibiotics (Dethlefsen et al., 2008;

Willing et al., 2011). Recent studies observed the change in the microbiota and an alteration of cytokine release in 3-week-old female C57BL/6 mice (Sun et al., 2019). Mice were given sterile water, enrofloxacin, vancomycin, or polymyxin B for 3 weeks. Their colons were removed and analyzed for histology, cytokine gene expression profiles, 16S rRNA sequencing, and metabolome analyses. Histology was largely unremarkable between samples. However, all three antibiotic treatments significantly up-regulated the gene expression of pro-inflammatory (*IFN-*γ, *TNF-*α, *IL-1*β, and *IL-6*) and anti-inflammatory cytokines (*IL-4*, *IL-17*, *IL-23,* and *IL-10*), but varied in fold-change depending on the treatment. Both vancomycin and enrofloxacin decreased the species richness and diversity indices of the colon microbiota. Further changes in fatty acid and amino acid metabolites were seen, which correlated with the presence of select microbial taxa.

In light of these illustrations we suggest that the oral cavity is no different from other ecological systems in the sense that it will build its microbial climax community based on its environmental components.

The Oral Cavity and Microbiome Analysis

Our oral microbiota is acquired at birth and over time from our primary care givers (Berkowitz and Jones, 1985; Lamell et al., 2000). The microbiome of the oral cavity has been studied since the 1960 where researchers began to appreciate that the supra and subgingival microbiota was composed of a complex consortium (Socransky et al., 1987; Fine, 2006). From the 1990s on there was a push to define all microorganisms present, and one could argue that the oral cavity was one of the original human-associated microbiomes to be characterized (Socransky and Manganiello, 1971; Socransky and Haffajee, 1991, 1994; Socransky et al., 1998). There was an appreciation for the fact that the oral cavity consisted of a consortium that is associated with disease, complicating the way in which infections were described. This new appreciation pushed for a revision of Koch's postulates, reformulated by oral microbiologists as Socransky revision of Koch's postulates (Socransky, 1979; Socransky and Haffajee, 1991).

Distinctive oral sites appear to be packed with commensals and these commensal can change from birth to senescence based on environmental changes in salivary flow and content, tissue rigor, hormonal conditions, diet, etc. Immediately after tooth brushing a succession of events occur on a tooth surface (Socransky and Manganiello, 1971). Pioneer colonizers, consisting mostly of Gram-positive bacteria, collect on the enamel surface in parallel arrays extending from the tooth surface (Kolenbrander, 2000; Kolenbrander et al., 2002, 2006; Li et al., 2004; Hojo et al., 2009; Esberg et al., 2020). These pioneers are succeeded by secondary and tertiary species all of which are commensal members of the oral microbiota ("normal" inhabitants of the oral cavity). The pioneer species, the hardiest of the oral microbial species, attach avidly to salivary-coated enamel surfaces (salivary pellicle) and form a resistant/adherent band of microbes. Astonishingly, these microbes were first identified in 1678 by Antonie van Leeuwenhoek as tiny "animalcules" (James, 1994; Lane, 2015). The pioneer primary colonizers followed by secondary and tertiary colonizers to a large extent make up our protective commensal microbiota, while also harboring potential pathobionts. Pathobionts are natural members of the human microbiota that have pathogenic potential under certain conditions (Mazmanian et al., 2008; Cugini et al., 2013).

Overall, there have been over 1,000 species identified that have the potential to make up the oral microbiome and we suspect that 70–100 species are present in any one individual (Dewhirst et al., 2010; Park et al., 2015; Xu et al., 2015). These numbers consist of the high abundance commensals as well as the lower abundance pathobionts and microbes of unknown function or role in disease. For disease either a physical assault or chemical biofilm induced-irritation, can result in a pathogenic biofilm that is characterized by out-growth of select species, which can give rise to many of the infections that arise in the oral cavity.

The Oral Microbiome: A Brief Historical Overview

Given that the oral microbes have been studied since the times of von Leuwenhoek, many of the early microbiologists were keen to study these bacteria and devised ways to study them *ex vivo*. One of the first intensive studies of oral microbes involved in disease was performed in the laboratories of Dr. Robert Koch by W. D. Miller, a visiting dentist from the United States. Miller, in a series of detailed experiments, clearly demonstrated the preference of oral microbes for carbohydrates and their relationship to acid production and caries (Miller, 1890). However, the main caries-culprit was first identified as *Streptococcus mutans* by Clarke, an English microbiologist in 1924. His description went largely un-noticed and therefore the relationship between these acid-loving, acid-producing oral microbes and caries was not totally accepted until the elegant experiments of Paul Keyes in 1964 (Clarke, 1924; Englander and Keyes, 1964). Keyes who was working on the effect of diet on caries made the serendipitous discovery that golden hamsters had caries while albino hamsters had none (Fitzgerald and Keyes, 1963; Englander and Keyes, 1964). After isolation of the microbes from the mouth of golden hamsters, inoculating pure cultures into the caries-free albino hamster, he showed how caries evolved. Keyes then did a series of experiments to show this microbe/host relationship in many well-designed experiments of experiment of which one in particular is worth highlighting. In this experiment, Keyes took albino pups delivered by Cesarean section in a germ-free chamber and housed these pups with golden hamster surrogate mothers, demonstrating that the caries-producing microbes could be passed from golden hamster mom to the albino pups and that the pups now showed carious lesions (Fitzgerald and Keyes, 1960; Keyes and Fitzgerald, 1962). In contrast, he took golden hamster pups and placed them in the cage with the albino mom. He showed that the golden hamster pups did not get caries and that the caries-producing organism could not be recovered from either the mom or the pups. These elegant experiments focused attention on *Streptococcus mutans* and led to many experiments attempting to show that caries was an infection caused by a specific microbes.

The microbiota associated with periodontal disease also served as an area of research. In the earlier studies, Rosebury and

colleagues in the 1930's and others (e.g., Kritchevsky and Seguin) made efforts to isolate microbes from periodontal pockets and showed how they provoked infections in a guinea pig groin model (Kritchevsky and Seguin, 1918; Rosebury et al., 1929, 1934). They concluded that a mixture of microbes was required and no single bacteria could provoke infection, but that a pathogenic quartet of microbes appeared to result in disease. These studies were challenged by Rosebury's graduate student J. B. MacDonald who pointed to *Bacteroides melaninogenicus* as the prominent pathogen related to the cause of periodontal disease (Macdonald et al., 1956). These controversies led to a series of alternative hypotheses developed by Dr. Walter Loesche, a former student of the MacDonald group (**Table 1**; Loesche, 1976), He introduced two hypotheses: (1) The Non-Specific Plaque Hypothesis (NSPH), and (2) Specific Plaque Hypothesis (SPH). The NSPH stated that disease was unrelated to specific microbes but rather was related to an accumulation of products derived from masses of microbes. In contrast, the SPH stated that a particular microbe provoked disease. These alternative hypotheses provided a window into the way in which infectious periodontal diseases could evolve. In time, these hypotheses evolved into the Ecological Plaque Hypothesis which states that the environment dictates the microbiota and must therefore be considered in terms of disease (Marsh, 1994). This theory is closest to how we understand microbial induced dental diseases today and in many ways illustrates a hypothesis that parallels the Damage/Response Framework (Casadevall and Pirofski, 1999).

EXAMPLES OF COMMENSALS AND PATHOBIONTS IN ORAL DISEASE

For dental disease to occur we propose that either a physical assault, chemical biofilm induced-irritation, and/or biological induced changes can result in a "pathogenic or disease promoting biofilm" (**Figure 1**). This putative "pathogenic" biofilm is characterized by outgrowth of select species, which we suggest can give rise to the most prevalent infections (periodontitis and caries) that arise in the oral cavity.

Periodontal Disease

In a microbiome at homeostasis there are delicate interspecies interactions driven by the maintenance of intricate physical and metabolic associations. These associations exert control over the host innate immune defenses in their effort to detoxify the environment. In periodontal disease there is a breakdown of this homeostasis that is characterized by the formation of a dysbiotic biofilm, plaque, and outgrowth of key pathobionts in the microbiome, which leads to host tissue destruction and ultimately, formation of periodontal pockets and bone loss. These chronic inflammatory diseases begin as reversible gingival inflammation (gingivitis), and if not managed, leads to advanced periodontal disease (Moore and Moore, 1994). Periodontitis results in increased bone resorption around the tooth and root area, which leads to eventual tooth loss. In chronic periodontal disease the host's oral microbiome shifts from a predominately Gram-positive healthy plaque biofilm to a pathogenic and primarily anaerobic dysbiotic consortium (Marsh and Zaura, 2017). The initiating events are poorly understood but it is likely a physical irritation that causes initial changes in the local environment, which allows for nutrient sources and an alteration in the local innate immune response. The Gram-negative pathobionts, while previously existing as low-abundance species begin to proliferate (Haffajee et al., 2008; Dewhirst et al., 2010; Uzel et al., 2011; Chen et al., 2018; Lamont et al., 2018; Curtis et al., 2020).

Physical Assaults That Can Result in Dysbiosis

Periodontal wounds can be caused by overhanging restorations, which can result in ulcerations of soft tissue leading to inflammatory changes in the underlying tissues. Early studies by Waerhaug (1956) clearly showed that roughly surfaced dental restorations placed below the gingival margin created histological changes in the underlying gingival tissue. In a dramatic illustration of this iatrogenic effect, one study took 9 dental students who needed mesio-distal inlay restorations (Lang et al., 1983). The restorations were place in the student's mouths and they were followed for tissue changes, inflammation, and microbiology over time (**Figure 2**). In one group, the restoration was perfectly fitted so that no tissue irritation would occur below the gum margin. The other group had a restoration that was designed to have a poorly constructed restoration with an "overhanging" metal margin that served to irritate the underlying tissue. The restoration was kept in place for 19–23 weeks, during which time the clinical condition was recorded as was bleeding on palpation every 2–3 weeks. The area below the gum was also sampled for predominant types of bacteria, which were determined by culture analysis of anaerobic bacteria. The restoration was then removed and redesigned such that the side that was perfectly fitted now had the "overhang" while the other side now had a well-fitted restoration, and the clinical and microbiological observations were continued as described. Bleeding on probing always was preceded by the presence of black-pigmented "Bacteroides" types of microbes, now known as Porphyromonads, microbes associated with gingivitis and periodontitis. These microbes were also associated with the overhanging margins, which were directly related to a change in the clinical condition, which led to the emergence of the Bacteroides type microbes. These changes document the potential for iatrogenic factors (poor dentistry) as an initiator of gingivitis and periodontitis causing a periodontal wound. Clinically this can be seen as an altered gingival sulcus now called a periodontal pocket.

This physical change can result in an alteration in microbial succession as compared to what typically takes place in an unadulterated environment. These changes can lead to bleeding and a change in the nutritional contents of the wound environment. Inflammation provides a specific set of nutritional factors, while bleeding provides red blood cells, hemin, and fibrin, factors that are useful nutritional and signaling metabolites for Bacteroidetes, Spirochetes, and Porphyromonads (Page and Schroeder, 1976). In the transition from health to disease the pocket deepens, anaerobiosis increases, available carbon sources decrease, and inflammation and pH changes occur which induce

TABLE 1 | Criteria for defining disease causation.

Criteria for defining disease causation	Date	Novelty	Focus	References
Koch's postulates	1893	Initial efforts to identify disease causation	Microbes	Koch, 1893
Loesche's criteria	1976	Efforts to examine microbial causes of dental diseases	Microbes	Loesche, 1976
Socransky modification of Koch's	1991	Efforts to include host in Koch's postulates	Microbes and Host	Socransky and Haffajee, 1991
Marsh ecological criteria	1994	Efforts to establish ecological influences on dental diseases	Microbes with ecological consideration	Marsh, 1994
Casadevall and Piroski Damage/Response	1999	Effort to understand host's participation in infectious diseases	Microbes in relationship to Host	Casadevall and Pirofski, 1999

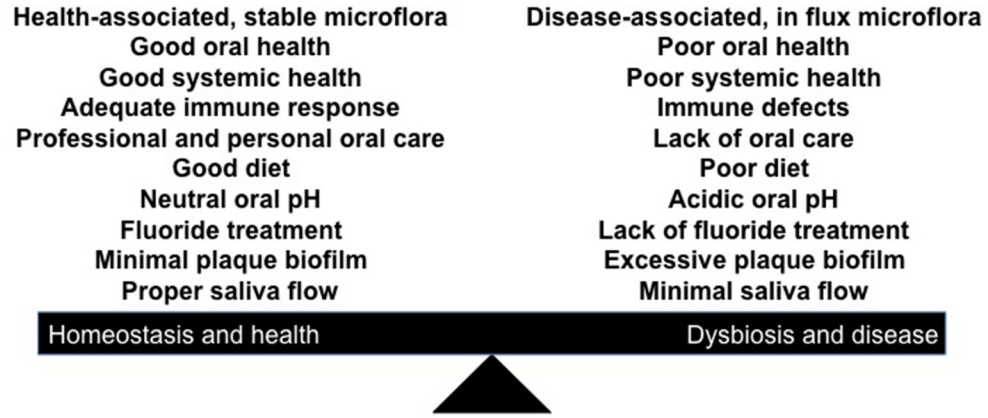

FIGURE 1 | Illustration of a shift in the homeostatic balance that favors dysbiosis in favor of disease. Influences on the right side that favor disease can include; physical, chemical, or biological influences that can disrupt the balance. Factors such as iatrogenic dentistry, nutrition (excess carbohydrates; type of diet), lack of salivary flow, changes in local pH, defects in enamel mineralization, inadequate immune responsiveness, leukocyte adhesion defects, etc. all can have a profound influence on dental disease at the local level.

temperature changes. These changes contribute to a fluctuating ecology in the context of the semi-delineated periodontal pocket environment (Loesche, 1993). When the intact delineated epithelial barrier is broached or ulcerated, oral bacteria can escape from the pocket and enter the blood stream (Fine et al., 1996). Here colonies from the subgingival biofilm invade the circulation. The first bacteria to appear in the circulation can aptly be termed pathobionts (or amphibionts), which now can invade a foreign territory, by moving through the blood stream, where they can settle on an irregular vascular niche. Now either single or multiple species that move away from their natural habitat course through the bloodstream and settle on damaged vessels or tissues (i.e., streptococci, Fusobacteria, *Aggregatibacter*, Bacteroidetes, porphyromonads, etc.).

Chemical Assaults That Can Result in Dysbiosis

A good example of the influence of antibiotics on the interference with natural microbiome homeostasis is illustrated by an experiment where specific classes of antibiotics were applied to tooth surfaces after thorough debridement (Loe et al., 1967). Since the sequence of events in early tooth related biofilm development was known it was possible to select antibiotics that would interfere with that progression. It was shown that streptococci in the oral cavity were known to be the first to interact with salivary-coated enamel surface immediately after debridement. After forming parallel arrays perpendicular to enamel the interstices between these parallel arrays was colonized by *Veillonella*, a Gram-negative facultative anaerobe. This was followed by a mixture of both Gram-positive and negative bacteria that formed a complex biofilm community in a 10-day period after abstinence from tooth brushing. Application of vancomycin, which inhibited Gram-positive bacteria, resulted in a thinner less dense biofilm; while application of polymyxin B, which inhibited Gram-negative bacteria, showed less of an overall effect, but the specific composition of the biofilm was significantly altered. The most dramatic effect was seen when tetracycline (a broad spectrum antibiotic) was painted onto cleaned tooth surfaces whereby an overall reduction in plaque biofilm was seen over this 5-day period of brushing abstention. This elegant but simple experiment demonstrated the potent effect of antibiotics on a change in the homeostatic balance in natural biofilm formation on tooth surfaces.

In clinical settings it has become apparent that a better understanding of antibiotics is imperative in the balance between health and disease (Sharma et al., 2019). Antibiotic sensitivity and resistance have to be taken into consideration

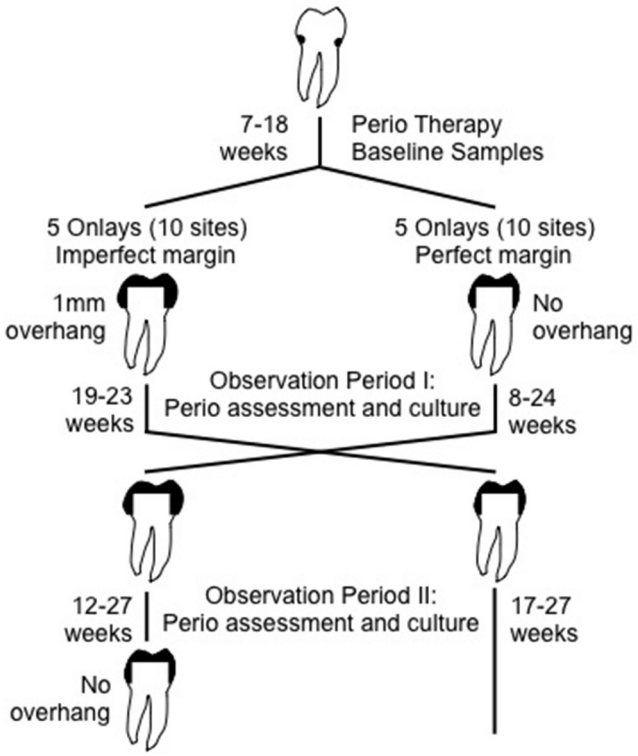

FIGURE 2 | Illustration of experiment showing how an overhanging dental restoration can influence homeostatic balance by influences on tissue integrity, inflammatory responsiveness etc. As such a physical change in the local environment that can produce a local inflammatory response that can lead to increased presence of red blood cells and cause an ecological shift in the microbial balance influencing the overgrowth of an anaerobic, heme dependent subgingival microbiota. Adapted from Lang et al. (1983).

(Marcinkiewicz et al., 2013). Effects of short-term antibiotic therapy have been questioned because the long-term clinical effect is not significantly superior to non-antibiotic therapy in efforts to reduce the pathogenic or dysbiotic microbiota (Hagenfeld et al., 2018).

Biological Assaults That Can Result in Dysbiosis

This example is seen mostly in what is thought to be genetically modified host response diseases, one example of which is Leukocyte Adhesion Deficiency (LAD). In LAD patients have a defect in leukocyte adhesion to endothelial cells resulting in defective transmigration of leukocytes into tissues (Hanna and Etzioni, 2012; Silva et al., 2019). Defects in CD18 expression result in a lowered B2 integrin expression on endothelial cells and leukocytes fail to migrate into the adjacent connective tissue. As a result, tissues are challenged by microbes that do not get removed, become infiltrated with these microbes, and patients succumb to progressive periodontal disease and tooth loss (Moutsopoulos et al., 2015). Recent evidence suggests that a dysregulated host response results in upregulation of IL-17 a bone modulating cytokine (Hajishengallis and Moutsopoulos, 2014, 2016). A less dramatic case of a biological assault can be seen in Localized Aggressive Periodontitis whereby a leukotoxin produced by *Aggregatibacter actinomycetemcomitans* can result in limited success for polymorphonuclear leukocytes (PMNs), lymphocytes and macrophages in the modulation of bacteria resulting in overgrowth of bacteria that could ordinarily remain under control. Thus host protective lymphocytes and leukocytes are affected at the local level producing an aggressive form of disease (Fine et al., 2013).

Caries

A second example of the prominent influence of the host on local oral disease can be seen in a description of caries. Caries (commonly called cavities) occur as a result of demineralization of enamel due to acid end-products generated by sugar consumption of oral bacteria that reside in dental plaque biofilm that collects on the enamel surface (Takahashi and Nyvad, 2011; Mira et al., 2017). Ultimately, there are key low-level acidogenic and aciduric pathobionts that reside in the cavity and due to a combination of tenacious biofilm formation, insufficient host innate response (i.e., saliva is not sufficient), and environmental factors (i.e., diet) these key organisms are allowed to survive in an otherwise inhospitable environment to other commensals. As the previously described chemically initiated environmental dysbiosis, these acidogenic and aciduric bacteria cause a dysbiosis of the local microbiome where only the most acid tolerant survive (Takahashi and Nyvad, 2011). In a healthy microbiome, the pioneer colonizers, in large part streptococci, form one's protective commensal microbiota and attachment sites for subsequent colonizers. Moreover, these organisms, in concert with other species create mutually beneficial environments for the colonizers (Kreth et al., 2005; Kolenbrander et al., 2006; Jakubovics et al., 2008a,b; Treerat et al., 2020). In rare instances these Streptococci species are responsible for extra-oral second site infections, such as endocarditis or abscess, hearkening back to Casadevall and Pirofski and the need to understand both the nature of the microbe and the host.

Here again we wish to stress the importance of homeostasis or more specifically the disruption of microbial and host homeostasis, which we term dysbiosis. As in the case of periodontitis we will divide these dysbiotic mechanisms into three categories; Physical, chemical, and biological factors that can influence a shift away from homeostasis to dysbiosis.

Physical Changes That Can Result in Dysbiosis

A physical constraint that encourages dysbiosis in the oral cavity occurs during tooth formation. The biting surfaces of molar teeth, called the occlusal surfaces, consist of pits and fissures, which are grooves and depressions in the top surface of teeth. The depth and tortuous anatomy of these surfaces play a role in how they contribute to the caries process. A deep pit or fissure in an occlusal surface can contribute to the accumulation of bacteria and forceful the packing of bacteria into these deep crevasses. As a result of this impaction, the bacteria residing in the depth of these recesses can rest in a protected domain and metabolize carbohydrates to produce acid and demineralize these surfaces (Fine, 1995). This process is

different than what happens in smooth surface decay of enamel where the microbes attach by means of protein adhesins found on the surface of the pioneer bacteria or to glycan receptors due to salivary binding to enamel surfaces. For occlusal decay bacteria such as *Lactobacillus* sp. that do not attach on their own, can establish a home in the occlusal pit without a need for adhesins. Here the bacteria can metabolize carbohydrates to reduce the biofilm pH to below 5.5 and thus produce decay (Fine et al., 1996). To counter-act this issue researchers have developed sealants made of thin plastic that fill in the occlusal pits and fissures to prevent the penetration of bacteria into these deep crevasses.

Another example of a physical change that can contribute to dysbiosis is in tooth root induced by exposure to bacteria due to gingival recession. The root is composed of a less mineralized cementum containing more irregular surfaces that becomes exposed due to a receding gum-line resulting from periodontal disease or trauma to the gingiva. Here we find an exposed cemental surface that has anomalies providing opportunities for colonization by bacteria, in particular those that would otherwise not colonize as they lack adhesive properties, to lodge in these areas and produce root surface decay (Takahashi and Nyvad, 2016). These conditions allow for bacteria to remove organic and inorganic material from root surfaces leading to physical changes that make the root more vulnerable to decay.

Chemical Changes That Result in Dysbiosis

Stephan showed that acid formation resulting from ingestion of carbohydrates resulted in a sharp drop in the biofilm pH immediately adjacent to the enamel surface (Stephan and Miller, 1943). It was shown that with repeated exposure to carbohydrates, members of the commensal microbiota that thrive at a low pH overgrew and these reputed causative microbes thrived at a low pH, which caused environmental selection that reduced diversification and changed microbial homeostasis (Marsh, 1994). Thus low pH favors the growth and survival of acid tolerant and acid producing microorganisms that thrive at a low pH, in this manner limiting diversification in this local patch or domain (Kianoush et al., 2014). Thus, carbohydrate-consuming species multiplied became profligate and produced lactic and formic acid as an end product of their metabolism (van der Hoeven and Franken, 1984; Duguid, 1985; Dashper and Reynolds, 1996). The patch or landscape in which the now low pH-favoring microbiota lives is typically influenced by salivary flow. By virtue of the buffering capacity of bicarbonate and other elements, the saliva buffers this sugar-induced pH decline returning the area to a neutral pH (Vila et al., 2019). However, in keeping with the Damage/Response Framework we now recognize that even in the case of infusion of sugar, that creates dramatic ecological changes favoring the outgrowth of acid tolerant/acid producing microbes, the microbiota is constrained by its environment, which is partially controlled overtime by salivary influences.

Biological Changes That Result in Dysbiosis

In caries as mentioned above, a critical element in the carious process is pH, and once the pH in a biofilm drops below 5.5 enamel demineralization begins (Dawes, 2003). The influence of saliva (a local Damage/Response regulator) on this process is critical due to its buffering capacity (Local Response), which if lacking can be catastrophic. This has been illustrated in several ways. In one case, in the absence of salivary flow, it is clearly shown that the pH drop will continue unaltered and disease or tissue damage will occur. An example of this occurs in patients who have had irradiated salivary glands that gives rise to reduced function and limited salivary flow resulting in excessive and uncontrollable caries on the side of the irradiated gland (Pinna et al., 2015). Without the buffering capacity of saliva a selective group of low pH acidophilic commensal microbes (microbial diversification has been reduced) have free reign. In addition to buffering, salivary antimicrobials that include but are not limited to lactoferrin, lactoperoxidase, lysozyme, IgA, as well as salivary flow itself, are severely reduced and therefore this landscape (the enamel surface) is much more vulnerable to the activity of low pH adaptive members of the commensal microbiota.

In a second simple demonstration of the importance of saliva, subjects who abstained from brushing for a short period in order to accumulate tooth associated plaque, were asked to rinse with a 10% sucrose solution (Abelson and Mandel, 1981). An antimony electrode was placed on tooth-surface-associated-plaque-biofilm in order to measure the pH of the biofilm in real time. A dramatic rapid decline in the pH was seen immediately after the sucrose rinse. Thus, the plaque/biofilm pH dropped from a neutral pH of 7.0 to <6.0; however, shortly thereafter salivary buffering forced the pH to rise again, protecting the enamel surface of the tooth from demineralization due to acid production. To demonstrate the prominence of host related saliva on this plaque pH effect, the salivary ducts of the subjects were blocked prior to the sucrose rinse, which prevented saliva from contacting the tooth associated biofilm. This interference with salivary flow allowed for the immediate pH drop but prevented salivary buffering and as such the subsequent pH rise failed to occur. As a consequence, the pH of the plaque/biofilm dipped below the 5.5 critical pH such that demineralization could take place. This simple experiment is another clear example of the prominence of the host in Damage/Response Framework determination of oral disease.

Summary of Causes of Dysbiosis and Dental Diseases

In summary, we have shown how classical common dental disease occur in the event of dysbiosis of the commensal microbiota initiated by an environmental stimulus that can cause a shift in the landscape ecology to the detriment of the host. In these examples there is no need for the addition of an exogenous pathogenic microbial species to cause disease. Rather classical dental "diseases" are more than likely to occur as a result of a shift in the activity and/or proportion of members of the commensal microbiota as a consequence of environmental changes. Thus, as in the case of most current infections, microbial interactions that have a damaging effect on the host are associated with the way in which the host manages the damage that can occur.

MICROBIAL AND IMMUNE INTERACTIONS IN HEALTH, INFLAMMATION, AND AUTOIMMUNE DISEASES

Brief Historical Prospective: General Immunology

Interest in immunology began in the late 1700's when small pox was raging as an epidemic in Europe. In 1796, Edward Jenner inoculated James Phipps with a scraping he obtained from a small pox lesion he removed from the arm of a dairy maiden who was infected by working with cows who had cowpox. This idea was derived from Benjamin Jesty who took scrapings from a cow (vacca) with a similar virus and inoculated his wife (Riedel, 2005). In 1875, Robert Koch inoculated the ear of a rabbit with blood of an animal that had anthrax. Shortly thereafter, Koch learned how to grow bacteria, validated the germ theory of disease, and began to establish the most advanced microbiology laboratory in the world (Williams et al., 2008).

Completely independently, Pasteur in 1879 began studying chicken cholera. In a serendipitous accident, Pasteur left nutrient broth intended to grow cholera toxin unattended in his laboratory over the summer. He then used the unattended broth, but left in his flask as an attenuated inoculum for chickens and found that they failed to get sick with cholera. In honor of Jenner, he called the process vaccination, but it was not until 1893 that Ehrlich identified the biological attributes as an anti-toxin material. In a contentious collaboration with von Behring and Shibasaburō, Ehrlich recognized that the anti-toxin for diphtheria was due to a soluble serum factor (Kaufmann, 2017).

In 1882, Eli Metchnikoff opened the door for research into white blood cells, phagocytosis, and innate immunity sparking the interest in cellular immunology (Gordon, 2008, 2016). Skipping ahead to 1939, Elvin Kabat, then at Columbia University, discovered that antibodies were gamma globulins (Kabat, 1983). In the 1960s, antibody structure was elucidated by Porter and Edelman, while Miller and Mitchell discovered B and T cell collaboration in functional antibody production in 1968 (Miller and Mitchell, 1968; Mitchell and Miller, 1968a,b; Raju, 1999; Sprent, 2017). A significant discovery of the activation of the innate immunity was made in the early 2000s by Beutler, Hoffman, and Steinman (Beutler, 2013).

Brief Historical Prospective: Immunology Related to Oral Disease

The work describing immunology in oral diseases paralleled the work done in medicine. The pathogenesis of dental infections received a great deal of attention when the emphasis shifted from pyorrhea as a local disease to a disease directly related to causes of systemic diseases of unknown etiology (Hunter, 1900). A link between oral infections and arthritis, colitis, heart disease, and cancer of unknown etiology was made and received a great deal of attention (Colyer, 1902). This theory was known as the "focal theory of infection" and was supported by several prominent dental and medical researchers and academicians, one of which was R. L. Cecil, author of the well-known Cecil and Loeb "Textbook of Medicine," first published in 1927 (Hunter, 1900, 1911; Billings, 1912; Cecil, 1929; Cecil and Angevine, 1938). During that period Rosenow, a prominent microbiologist, performed scientific experiments using animal models in efforts to show how microbes from the oral cavity provoked systemic infections (Rosenow, 1919, 1930). After several instances related to "extreme treatment" of human "dental infections," whereby treatment resulted in extraction of all teeth, it was determined that the extreme treatment failed to result in any changes in overall systemic health. This approach put an end to the belief in this theory and the practice of "extreme treatment" was fortunately abandoned.

As for vaccinations related to pyorrhea, Beckwith et al. (1929) made efforts to inoculate animals with organisms isolated from pyorrheatic pockets (Beckwith et al., 1925, 1929). He compared reactions to heat attenuated plaque derived from humans to boiled plaque samples then inoculated into humans and rabbits. Several of the animals died in the heat-attenuated samples as opposed to the boiled samples suggesting some semi-viable toxic material (Beckwith et al., 1929). A series of studies were also initiated by Rosebury to develop caries vaccines although the investigators focused on lactobacillus as opposed to streptococci (Rosebury et al., 1929, 1934).

Major contributions to the study of oral immunology were made by the Alabama dental research group consisting of Drs. J. McGhee, Mestecky, and Michalek, also involving Dr. Per Brandtzaeg and Frederick Kraus. As prominent contributors to our understanding of the common immune mucosal system (CIMS), the group clearly illustrated the unification of IgA pathways when antigens were provided via vaccines to mucosal surfaces as compared to intramuscular inoculations (Mestecky et al., 1972, 1978, 2008; McGhee et al., 1987; Moldoveanu et al., 1995). Studies from this group revolved around development of a caries vaccine against *S. mutans,* which provided a unique understanding of mucosal immunity and ultimately showed differences in IgG, IgM, and IgA responses. Kiyono, also part of this group, provided a new method for separating dendritic cells and macrophages as antigen presenting cells in Peyer's patches and showed that oral delivery of antigens produced Ig isotype subset of Th2 type helper cells that induced IgA responsiveness (Kiyono and Fukuyama, 2004; Kiyono and Azegami, 2015).

Recognition of Pathogens by the Innate Immune System

The vital observation that the induction of a strong immune response against purified proteins was dependent on the presence of microbial constituents, such as killed bacteria or bacterial extracts, famously called "the immunologist's dirty little secret" by Janeway (1989), gave birth to the term **adjuvant** (which in Latin means *adjuvare*, for "to help"). In the absence of infection, it is clear that adjuvants are partially required to activate innate receptors on sensor cells to aid T cells (lymphocytes). Sensor cells that detect infection and drive the production of inflammatory mediators include macrophages, neutrophils, and dendritic cells. Such cells express a number of innate recognition receptors that enable them to detect pathogens or the damage caused by them. These receptors are known as pattern recognition

receptors (PRRs) and recognize simple molecular structures termed pathogen-associated molecular patterns (PAMPs), also called microbe-associated molecular patterns (MAMPs), which are components of many microorganisms, but not of the body's own cells (Yu et al., 2017; Negi et al., 2019). PAMPs come in various flavors and are expressed by different classes of bacteria, which engage several pattern recognition receptors (PRRs) (**Table 2**).

While the first line of innate immune defense involves detection of PAMPs or MAMPs, danger-associated molecular patterns (DAMPs) are endogenous factors released upon cellular damage or tissue disruption (Kay et al., 2019). DAMPs released from oral and salivary tissue play an important role in progression of inflammatory and autoimmune disease. The signaling pathways of PAMPs and DAMPs intersect in the manifestation of diseases of the oral cavity, particularly in periodontal disease, oropharyngeal candidiasis, and Sjögren's Syndrome (De Lorenzo et al., 2018; Kay et al., 2019).

Immunologic Signals Induced by Pathogen Recognition

Chemokines are chemotactic cytokines whose function is critical for the positioning of immune cells in tissues. They control release of innate immune cells from the bone marrow, as part of normal homeostasis, and as a result of infection and inflammation. They play a critical role in guiding innate immune effectors out of the circulation and into sites of injury or inflammation. In doing so, chemokines promote, and coordinate interactions between the innate and adaptive immune systems, thus ensuring optimal adaptive immune responses (Hao et al., 2010; Sokol and Luster, 2015). Neutrophils are the first cells to arrive at sites of infection, and they provide a front line of defense against bacterial infection. While most bacteria are readily killed by neutrophils, some bacterial pathogens have the capacity to circumvent destruction by these host leukocytes (Teng et al., 2017; Kobayashi et al., 2018). There is an elaborate cellular and cytokine presence at the gingival tissue interface and supporting oral mucosa, where an increased amount of neutrophils are recruited to the gingival crevice during inflammation, such as conditions found in gingivitis or periodontitis (Dutzan et al., 2016; Moutsopoulos and Konkel, 2018). Under normal conditions these neutrophils play an important role in microbial surveillance as well as in coordinating the overall immune response, in order to maintain oral health.

Evidence suggests that bacteria in biofilms, including those found in the supra- and subgingival plaque biofilm, are more resistant to the phagocytic activities of neutrophils and macrophages than non-biofilm bacteria (Ebersole et al., 2017; Liu et al., 2017). As a result, the sentinel cells that mediate the first line of the adaptive immune response, comprising dendritic cells, macrophages and mast cells, are called in for battle, scanning for the foreign invaders. The initial response is to destroy the invaders, followed by distress signals that are sent via cytokines and chemokines that recruit reinforcement of other effector cells to eliminate the remaining threat.

The adaptive immune system of the gastrointestinal tract has unique features that distinguish it from those of other organ systems. The most important adaptive immunity in the gut is humoral and is geared toward keeping the microbes of the lumen under control. This property is mediated by dimeric IgA antibodies, which are secreted into the lumen of the gut or found in the colostrum of mother's milk ingested by infants (Macpherson et al., 2018; Bryant and Thistle, 2020). IgA in the gut is critical in preventing commensals and pathogens from invading via the epithelial barrier of the mucosa. The preponderance of IgA in mucosal secretions is due to the fact that activated B cells in the gut undergo class switching to IgA producing B cells, which home to the gut. Cell mediated immunity against gut microbes are mediated by helper T cells, of which Th17 cells are the most abundant, even though Th1 and Th2 cells are also found. Regulatory T cells (Tregs) are most committed to maintaining tolerance to food antigens (Tordesillas and Berin, 2018), and to commensal microbial antigens (Nutsch and Hsieh, 2012).

Resident macrophages and dendritic cells are normally present in the gingiva and are important in defending the tissue barrier against bacterial insult. In response to microbial dysbiosis, the numbers of these cells increase (Delima and Van Dyke, 2003). In health, lymphocytes in the gingiva comprise few B cells and more prominent T cells. During disease, various B-cell and T-cell subsets increase significantly, where Th17 cells may promote pathogenesis. While little is known about specialization of Treg cells in the gingiva, it is clear that Tregs play critical roles in maintaining periodontal homeostasis (Glowacki et al., 2013; Moutsopoulos and Konkel, 2018). Unlike homeostatic oral Th17 cell accumulation, in a commensal-independent and IL-6-dependent manner, periodontitis-associated expansion of Th17 cells was dependent upon the local dysbiotic microbiome and required both IL-6 and IL-23 (Silva et al., 2015; Dutzan et al., 2018). Th17 cells secrete the IL-17 cytokines, which have pro-inflammatory activities in common with IL-1β, TNFα, and IL-22, and are important for immunity against extracellular bacteria (Miossec, 2009). Th17 cells are involved in the pathogenesis of several autoimmune and inflammatory disorders; in fact three IL-17 inhibitors have been approved for the treatment of psoriasis, psoriatic arthritis, and ankylosing spondylitis (Beringer and Miossec, 2019). As it relates to the oral cavity, IL-17A has been shown to stimulate the development of osteoclasts (osteoclastogenesis) in the presence of osteoblasts (Zhang et al., 2011), and expression of IL-17 has been observed in gingiva from patients with periodontitis (Cardoso et al., 2009). In an *A. actinomycetemcomitans*-induced rat model for periodontal disease prior to onset of bone resorption, upregulation of IL-17 in CD4+ T cells (2.8-fold) and B cells (2-fold) in lymph nodes from *A. actinomycetemcomitans*-infected rats was observed, as compared to control rats (Li et al., 2010; Tsiagbe and Fine, 2012).

The Th17/IL-17 response has been investigated as a therapeutic target. Resolvin E1 (RvE1), a product of the ω-3 polyunsaturated fatty acid eicosapentaenoic acid is known to be a potent pro-resolving lipid mediator that prevents chronic inflammation, osteoclastogenesis, and bone resorption by inhibiting IL-17-induced RANKL expression in osteoblasts

TABLE 2 | Pattern recognition receptors (PRRs) and the associated pathogen-associated molecular patterns (PAMPs).

	PRR	PAMP	References[1]
Gram positive	TLR2, NOD2	Peptidoglycan (PGN)	a, d
	TLR2	Teichoic acid (TA)	b, c, d, e, i,
	TLR2/6	Lipoteichoic acid (LTA)	e, f, g
	TLR5, NAIP5, NAIP6, NLRC4	Flagella	d, zc
Gram negative	TLR4	Lipopolysaccharide (LPS)	d, h
	TLR2, NOD1, NOD2	PGN	d, j
	TLR5, NAIP5, NAIP6, NLRC4	Flagella	d, h
	TLR2	Porins	k, l
	NAIP2, NLRC4	Rod protein of Type III secretion system (T3SS)	m, n
Genus-specific			
Mycobacteria	TLR2, MARCO, MINCLE	Trehalose dimycolate (TDM)	o, p
	TLR2/4	Mycolic acid (MA)	q, r
	TLR2	Lipoarabinomannan (LAM)	s, t, u
	TLR2	Arabinogalactan (AG)	v
	TLR2, NOD2	Peptidoglycan (PGN)	w
	TLR4	Phosphatidylinositol mannose (PIM)	x
Mycoplasma	TLR2/6	Macrophage activating lipopeptide M161 antigen	d, y
Other	TLR9	CpG-DNA	z, za, zb

[1]*a, Rosenzweig et al. (2011); b, Ribeiro et al. (2010); c, Kumar et al. (2013); d, Kumar et al. (2011); e, Takeuchi et al. (2000); f, Krutzik et al. (2003); g, Takeuchi et al. (2000); h, Takeuchi et al. (2002); i, Echchannaoui et al. (2002); j, Clarke et al. (2010); k, Singleton et al. (2005); l, Mukherjee et al. (2014); m, Kofoed and Vance (2011); n, Karki et al. (2018); o, Bowdish et al. (2009); p, Martinez et al. (2016); q, van Crevel et al. (2002); r, Harding and Boom (2010); s, Strohmeier and Fenton (1999); t, Hook et al. (2020); u, Gilleron et al. (2003); v, He et al. (2019); w, Girardin et al. (2003); x, Abel et al. (2002); y, Nishiguchi et al. (2001); z, Peter et al. (2009); za, Adamus and Kortylewski (2018); zb, Hausmann et al. (2020); zc, Lai et al. (2013).*

and RANKL-induced osteoclast differentiation (Funaki et al., 2018). Its activities have made RvE1 a new therapeutic target of rheumatoid arthritis. Additionally, resolvins hold promise for treatment of periodontal disease and other inflammatory diseases, including type 2 diabetes and cardiovascular disease (Van Dyke, 2017).

Mucosal tissues, which are colonized by a dense and diverse microbiota of commensal bacteria, are often the initial sites of interaction with pathogenic microorganism (D'Aiuto et al., 2004; Ebersole et al., 2017). Macrophages efficiently recognize unique classes of microorganism-associated molecular patterns (MAMPs), which facilitate the avid uptake of the microbes by pattern recognition receptors (PRRs) (Lauvau and Glaichenhaus, 2004; Ebersole et al., 2017). In the "classical activation" (M1), the macrophages display an inflammatory function that leads to cytotoxicity, tissue injury, and fibrosis (Locati et al., 2013). The differentiation into M1 macrophage phenotype relates to host-derived IFN-γ, as an autocrine or paracrine factor, and lipopolysaccharide (Labonte et al., 2017). The "alternative activation" (M2a,b) process is driven by IL-4 and IL-13, which can be autocrine or paracrine, and is immunomodulatory in its control of tissue repair and cellular regeneration (Mantovani et al., 2013). Macrophage activation plays a large role in periodontal diseases. The outcome of antigen recognition is dependent on which functional subpopulations of macrophages are engaged. The oral pathogens *P. gingivalis* and *A. actinomycetemcomitans* were observed to induce M1-type cells, whereas oral commensal bacteria primarily elicited macrophage functions consistent with an M2 phenotype (Huang et al., 2016). The presence of relatively more M1 macrophages, compared to M2 macrophages in gingival tissue may be responsible for the development and progression of inflammation-induced tissue destruction, and modulating macrophage function may be a potential strategy for periodontal disease management (Zhou et al., 2019).

Immature dendritic cells (i.e., Langerhans's cells), which are endowed with the ability to capture antigen, are normally located in the gingival epithelium, while mature dendritic cells, which specialize in antigen presentation, tend to infiltrate specifically the lamina propria of the gingiva, an area enriched for CD4+ T cells (Jotwani et al., 2001). While much work is still needed to elucidate the role of dendritic cell subsets in periodontal disease, it is established that immature dendritic cells were more prevalent in aggressive periodontitis than chronic periodontitis (da Motta et al., 2016).

Molecular Mimicry and Its Pathologic Consequences

The process of aging is characterized by quantitative modifications of the immune system, described as "immunosenescence," which leads to increased susceptibility to infections, neoplasias, and autoimmune manifestations, primarily due to persistent antigenic stimulation and/or stress responses across the life span (Weng, 2006; McElhaney et al., 2012; Ebersole et al., 2016; Mancuso et al., 2018). This diminution in the ability to withstand antigenic stimuli or stressors is often accompanied by enhanced proinflammatory state, known as "inflammaging" (Ebersole et al., 2016; Fulop et al., 2018). This enhanced proinflammatory state is shared by the elderly who age with minimal morbidities (i.e., no comorbidities) and those who do not (Mari et al., 1995; Ebersole et al., 2016). This observation led to the hypothesis that a threshold exists beyond which an individual is driven toward unsuccessful aging (Shanley et al., 2009). The mechanisms that underlie inflammaging are not well-elucidated. One explanation put forward is that it is driven by changes in the numbers and frequencies of innate immune cells, or alteration in the expression of or signaling via PRRs (Baggio et al., 1998). A generalized age-associated decreased in toll-like receptor (TLR)-induced cytokine production has been observed (Canaday et al., 2010). With respect to periodontal

disease, age-related decline in IL-6 induction in macrophages by *P. gingivalis* has been observed (Liang et al., 2009). In some individuals, age-related decline in TLR-dependent expression of costimulatory molecules CD80 and CD86 has been observed in monocytes, myeloid dendritic cells, and plasmacytoid dendritic cells (Qian et al., 2011; Sridharan et al., 2011; Ebersole et al., 2016).

Knowledge about age-related changes in the composition and phenotype of cells in the periodontium, which lead to alveolar bone resorption, gave birth to the concept of "osteoimmunology" (Feng and McDonald, 2011; Schett, 2016; Terashima and Takayanagi, 2018; Okamoto and Takayanagi, 2019). Age-related increases in RANK expression on osteoblast progenitors and RANKL expression in supporting mesenchymal stromal cells has been noted to result in a pro-osteoclastic environment, which potentially promotes bone resorption (Chung et al., 2014; Ebersole et al., 2016). While age-related enhancements in proinflammatory cytokines, such as prostaglandin E2, TNF-α, IL-1β, IL-6, and IL-17 are suggested to play significant roles in in enhancing osteoclastogenesis (Boyle et al., 2003; Ebersole et al., 2016), other molecules such as IFN-β, IL-4, IL-10 and chemokine axis of CCR4 and CCL22 dampen bone loss by a molecular feedback mechanism (Araujo-Pires et al., 2015; Ebersole et al., 2016).

Molecular mimicry of host proteins is an established strategy adopted by bacterial pathogens to interfere with and exploit host processes. Mimics within pathogens arise via two evolutionary mechanisms: (1) pathogen genomes can obtain host genes directly through lateral transfer or (2) through convergent or parallel evolution of a pathogenic protein toward resemblance of a host protein (Koonin et al., 2001; Stebbins and Galán, 2001; Elde and Malik, 2009; Doxey and McConkey, 2013). The Gram-negative bacterium *Helicobacter pylori* is a common bacterial pathogen that is responsible for widespread gastrointestinal morbidity worldwide and employs of a number of mechanisms of molecular mimicry (Kamboj et al., 2017). *H. pylori* colonizes the gastric mucosa in humans, and increases the risk of serious diseases such as gastric and duodenal ulcers, stomach cancers, and mucosa-associated lymphoid tissue lymphoma. *H. pylori* employs antigenic mimicry and possible deleterious effects due to the induction of immune response to the components common to these bacteria and the host (Chmiela and Gonciarz, 2017). *H. pylori*-related growth retardation in children is a noted phenomenon, however, it is poorly understood. Gastrointestinal microbiota, including *H. pylori* may produce antigens that mimic appetite-regulating peptides, resulting in the production of auto-antibodies, which modify the actions of key appetite-regulating peptides, such as alpha-melanocyte-stimulating hormone (α-MSH) (Fetissov et al., 2008). Polymorphisms of the host interleukins, including IL-1β, TNF-α, and cyclooxygenase-2 (COX2) have been suggested to increase the risk of infection and its severe consequences (Machado et al., 2003).

There is increasing support to the idea that gut dysbiosis, with an imbalanced state of microbiota, might be associated with the pathogenesis of autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), ankylosing spondylitis (AS), and inflammatory bowel disease (IBD) (Kim et al., 2016). Tetracycline derivatives, such as doxycycline and minocycline, are safe and moderately effective disease modifying anti-rheumatic drugs in the treatment of early RA patients (Smith et al., 2011; Kim et al., 2016). Probiotics, which are live microorganisms that confer health benefits to the host and which have the potential to maintain a healthy microbial balance in the gut, have been tested. *Clostridium* consortium, *F. prausnitzii,* and *Bifidobacterium* have been tested to ameliorate IBD in the colitis model by inducing Treg cells and anti-inflammatory effects (Atarashi et al., 2011; Kim et al., 2016).

CURRENT AND FUTURE METHODOLOGIES FOR THE EVALUATION OF HEALTHY AND DYSBIOTIC COMMUNITIES

The imbalance in the composition of oral microbiome can be directly linked to disease conditions. However, because of the overlap in the microbial communities, the difference between the healthy and disease states cannot be solely explained by the differences in the microbial composition. Thus, additional elements such as the functional activities of the microbiomes are needed to fully characterize and define the dysbiotic process. In this regard, recent omics studies have analyzed gene expression changes to analyze the functional activities and will be discussed below in detail.

Omics Technologies

The omics approach encompasses various technologies applied to fields of research including genomics (and epigenomics), transcriptomics, proteomics, and metabolomics. The ultimate goal of these approaches is to design diagnostics to predict an individual's risk to develop disease and/or to determine whether or not specific treatments are suitable for the individual patient. A genomics approach has been widely used, especially in cancer diagnoses. Recently, genome-wide association studies (GWAS), and next generation exome and genome sequencing data have amassed a large set of DNA sequence variants that can be associated with diseases in humans (Olivier et al., 2019).

Genome-Wide Association Studies and Periodontitis

Although GWAS have had modest success, (Offenbacher et al., 2016) supplemented the clinical data with biological intermediates of microbial burden and the local inflammatory response [gingival crevicular fluid (GCF) IL-1β] to derive periodontal complex traits (PCTs) for chronic periodontitis. Six PCTs were derived. PCT1 (loci CLEC19A, TRA, GGTA2P, TM9SF2, IFI16, and RBMS3) was characterized by a uniformly high pathogen load; PCT3 genetic variants of diacylglycerol kinase and inositol polyphosphate phosphatase, which are critically involved with regulating neutrophil function; and PCT5 (loci *SLC15A4*, PKP2, and SNRPN) were dominated by *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. Several studies aiming to explore the distinct gene expression profiles and pathways unique to periodontitis

have indeed confirmed the anticipatory differential expression involved in inflammation or bone resorption (Zhang et al., 2020). For example, pathways involved in cytokine and chemokine activities, B-cell receptor signaling, and defense and immunity proteins in both innate and adaptive immune responses, were reported to be among those most upregulated in periodontic gingival tissues when assessed using RNA sequencing.

It is unclear at present, despite much thought in to the subject of "Infectogenomics," as to how host genetics helps to shape the healthy or dysbiotic microbiome in periodontitis (Kellam and Weiss, 2006). Recently, groups have been exploring how host genetic variants affect the colonization of bacteria, which affects the genetics-associated dysbiosis (Nibali et al., 2014, 2016). In a model proposed to explain this dysbiosis, it has been suggested that single-nucleotide polymorphism (SNP) variants may compromise genes that are associated with host pathways of bacterial sensing and recognition (Zhang et al., 2020). The advent of 16S ribosomal RNA gene sequencing has allowed the evaluation of phylogenetic relatedness of bacterial species. If the bacterial sensing and recognition pathways are affected and result in a dysbiotic biofilm, could this lead to a shift in the microbial taxa wherein the health-associated species are suppressed and periodontitis-associated bacteria dominate subgingival communities (Diaz et al., 2016)? Using 16S pyrosequencing of the microbiome of periodontitis patients and healthy controls, Griffen et al. (2012) reported the enrichment of 123 species abundant in periodontitis compared to only 53 species in the healthy state. This microbial shift in the proportion of existing species might result in a succession process of emergence of disease bacteria without replacing the health-associated species. As described below, it is possible that the shift in microbiota may also result in a metabolic shift in periodontitis patients.

Metatranscriptomics

This omics method is a technology designed for the functional characterization of microbiomes using microarray or RNA sequencing technologies that reveals the taxonomic composition and active functions of a complex microbial community. It is unlike metagenomics, which shows the microbial DNA composition, and with an added bonus over previous methods since there is no PCR or primer/probe bias (Socransky et al., 1994; Kumar and Gupta, 2003; Colombo et al., 2009). With the well-characterized human microbiome, metatranscriptomics has facilitated many studies about the dysbiosis and disease. Discussed below are some such studies, which have helped us to understand the functional characterization of the microbiome.

Metatranscriptome and Caries

In an earlier study, focusing on the metatranscriptome during biofilm formation and before and after meal ingestion, the authors set out to identify the transcriptionally active portion of the supragingival microbial community in relation to the metagenome (Benitez-Paez et al., 2014). Clearly, from one individual the three most abundant genera from the metatranscriptome analysis of 24-hr plaque corresponded to *Actinomyces, Corynebacterium,* and *Neisseria,* whereas the most abundant genera from the metagenome were *Veillonella, Streptococcus,* and *Leptotrichia*. In the same study, the authors also analyzed the gene expression during plaque development and compared the genera during early (6–12 h) and mature biofilm (24–48 h) formation times. Interestingly, there was significantly ($p < 0.0118$) fewer genera in the early compared to the mature biofilm. Both *Streptococcus* and *Actinomyces*, known partners of coaggregation were found predominantly in the early time point (Kolenbrander et al., 1983). The bacterial activity during biofilm formation in healthy subjects was found to be person-specific. While in the early plaque samples, up regulation of genes involved in the carbohydrate metabolism, energy, vitamins, and amino acids were observed, in the mature biofilm the functional categories included ABC transporters, chemotaxis, and pilus assembly. One aspect of the study suggested that at least in some individuals there was no difference in the microbiota before or after a meal thus maintaining homeostasis and promoting dental health (Benitez-Paez et al., 2014).

Metatranscriptome and Periodontitis

The microbiota associated with periodontitis, a disease resulting in part from polymicrobial synergy and dysbiosis, has been classified into color coded complexes depending upon their accumulation in the periodontal pocket (Socransky et al., 1998; Lamont and Hajishengallis, 2015). Although the mean species (beta) diversity changes drastically between the disease and healthy states, higher alpha (average species) diversity and biomass appear to be associated with the disease community when the subgingival plaque was analyzed from chronic periodontitis patients and healthy subjects (Abusleme et al., 2013). In spite of the emergence of dominant taxa, the original health-associated community did not get replaced. Subsequent statistical analysis of two existing data sets, however, revealed a reduced alpha diversity associated with disease (Duran-Pinedo et al., 2014b; Yost et al., 2015; Ai et al., 2017). In a recent metatranscriptomic analysis, notably, the functional comparisons between healthy and generalized aggressive periodontitis sites revealed that upregulation of lysine fermentation, histidine degradation, and pyruvate metabolism is common among diseased individuals (Jorth et al., 2014). However, three metatranscriptomic surveys into the metabolic activity of chronic periodontal disease progression provided further insight that the conservation of the community functionality rather than the specific microbial effecters of disease exists (Duran-Pinedo et al., 2014a,b; Yost et al., 2015). A very recent work, examining the existing metatranscriptome datasets (Duran-Pinedo et al., 2014b; Jorth et al., 2014; Yost et al., 2015) to identify the commonly differentially expressed transcripts from both chronic and aggressive periodontitis patients and potential underlying RNA regulatory mechanisms behind the metabolic shifts, has revealed that many ncRNAs (both known and putative) may facilitate the metabolic shifts associated with periodontitis (Ram-Mohan and Meyer, 2020). Some of the notable highlights of this new analysis are: (1) Only a fraction of the differentially expressed transcripts originate from red/orange complex; (2) The differentially expressed transcripts from these Red/Orange complexes show the greatest magnitude of change;

(3) Enrichment of genes generally grouped into the biological process of localization could be important in the establishment of red complex pathogens in the periodontal pocket to drive pathogenesis; (4) Most metabolic pathways enriched in disease state have multiple contributing species fulfilling the metabolic niche; and (5) By applying *de novo* pipelines on the differentially expressed genes, the authors identified several putative sense and anti-sense regulators of bacterial ribosomal proteins that could be associated with periodontitis. Additionally, seven novel antisense ncRNAs targeting ribosomal proteins may be involved in maintaining ribosomal protein stoichiometry during the disease associated metabolic shift (Ram-Mohan and Meyer, 2020). In summary, metatranscriptomics analyses suggest a common shift in metabolic signatures in disease vs. healthy communities with up-regulated processes including pyruvate fermentation, histidine degradation, amino acid metabolism, and TonB-dependent receptors.

Proteomics

Simply put, proteomics is the methodology wherein proteins are identified in a sample. The key technology in proteome analysis is mass spectral analysis, although classical approaches have also utilized gel electrophoresis, liquid chromatography, and microarray. Mass spectrometry (MS)-based proteomics is a large-scale, high-throughput, systematic study, allowing for the comprehensive characterization of total protein in a sample, even with a limited sample volume or mass. Recent advances in the mass spectrometry platform utilize selective, multiple, and reaction monitoring to quantify precisely and reproducibly even low abundance proteins, which makes the technology even suitable for clinical use (Uzozie and Aebersold, 2018). Many of the current analyses also include protein-network analysis for enhancing the analytical outcome.

Proteomics and Caries

For caries research, the proteome of early pellicle (3 min) samples isolated from 12 caries-free and 12 caries-active patients was analyzed (Trautmann et al., 2019). Among the 1,188 proteins identified, there were 68 that were ubiquitous regardless of disease state. Quantitative analysis suggested that 23 proteins are potential caries-specific biomarkers. A higher extent of protein identifications might facilitate the future large-scale analyses to identify discrimination factors for the development of caries susceptibility tests (Trautmann et al., 2019).

In another study involving children with and without caries, using multiple reaction monitoring, unstimulated saliva from three groups of 10 children each with no, low, and high caries were analyzed (Wang et al., 2018). Among the 244 differentially expressed proteins, the authors selected 53 proteins, including mucins, histatin 1, cystatins, and basic salivary proline-rich protein 2, for further verification using multiple reaction monitoring assays. An interesting conclusion from this study is that there might be synergistic action among the proteins for caries resistance and for carcinogenicity. Unlike other proteome studies, this type of analysis might be used in the development of biomimetic, therapeutic peptides with preventive benefits for childhood caries (Wang et al., 2018).

Proteomics and Periodontitis

Recently, proteomics approaches have been applied to the diagnosis of periodontitis through the identification of protein biomarkers by comparing complete protein profiles in health vs. disease conditions using tandem mass spectrometry (Antezack et al., 2020). This study was designed to evaluate the protein profiles exclusive of the potential risk factors such as age, gender, hypertension, smoking habits, or diabetes by using a principal component analysis of top-ranking peaks and epidemiological data from a medical questionnaire from 141 subjects (67 periodontal and 74 control subjects). Samples from saliva, GCF, and plaque were used in the analysis, which showed that the composition in these samples reflected important differences in correlation with disease population (Papapanou et al., 2018). Although GCF showed a strong ability to distinguish periodontal patients with a sensitivity of 79.6 (0.188) and a specificity equal to 75.7 (0.195), saliva can be used as a simple diagnostic fluid for screening potential periodontal patients [sensitivity = 70.3% (0.211) and specificity = 77.8% (0.165)]. While other studies based on bacterial composition 16S rRNA analysis have shown *Prevotella* to be overabundant in healthy subjects, salivary microbiota such as *Porphyromonas, Tannerella, Desulfobulbus, Eubacterium, Phocaeicola,* and *Mogibacterium* were associated with periodontitis patients (Chen et al., 2018). One of the major outcomes of this mass spectral study is that periodontal diagnosis does not depend on a unique biomarker and that patients who are symptom-free can be screened at the early stage of disease without the need of clinical measurements such as pocket depth, plaque index and radiography (Papapanou et al., 2018). Thus, the mass spectroscopy analysis could be used as a simple, non-invasive, and rapid screening method on a large population within a short period of time to results (24–48 h).

In another study, Hartenbach et al., using a hybrid mass spectrometer LTQ Orbitrap Velos for proteomic analysis to obtain greater resolution, mass accuracy and sensitivity, were able to improve the qualitative and quantitative analyses of the salivary proteome (Hartenbach et al., 2020). The authors showed that a large range of salivary proteins with protective functions and associated with oral homeostasis were down-regulated in chronic periodontitis patients compared to individuals presenting periodontal health. In contrast, very few specific proteins such as salivary acidic proline-rich phosphoprotein, a submaxillary gland androgen-regulated protein, histatin-1, fatty acid binding protein, thioredoxin, and cystatin-SA were increased in chronic periodontitis or related to periodontal tissue destruction and inflammation. It is possible that a decrease of several proteins related to innate immune response and tissue integrity may determine the disease profile. These differences in salivary proteome profiles between periodontal health and periodontitis may contribute to the identification of disease indicators, and to the improvement of periodontal diagnosis and treatment.

What is the clinical relevance of these studies? While it could be argued that routine MS analysis might be useful, this technique also requires specialists for the analysis and might be time consuming. However, identifying a small subset of reliable changed proteins from these proteomic studies could lead to

consistent identification of salivary proteins that could then be used in a chair-side test. In this regard, some recent studies have shown that S100A8 and S100A9 in GCF and saliva could be candidate biomarkers for periodontitis (Preiano et al., 2016; Shin et al., 2019). Fine et al., has shown that MIP1-α levels were 50-fold higher in aggressive periodontal patients' in saliva and can be used as a predictive marker for site-specific disease progression (Fine et al., 2009, 2014). MIP1-α has also been shown to be predictive in chronic periodontitis patient's GCF or saliva, as well as being one of a set of cytokines that influence the bacterial composition at the periodontal pocket, indicating a potential relationship to dysbiosis (Al-Sabbagh et al., 2012; Syndergaard et al., 2014; Zhou et al., 2017). Interestingly, this potential biomarker is elevated in many inflammatory and disease conditions that exhibit bone resorption, such as periodontitis, multiple myeloma, Sjögren syndrome, and rheumatoid arthritis (Bhavsar et al., 2015). Thus, a rapid, point-of-care test-kit using salivary MIP1-α, S100A8, and/or S100A9 could be a practical tool for diagnosis and reducing the risk of periodontitis and promotion of periodontal health (Buzalaf et al., 2020).

These studies provide a small sample of the potential of proteomics in diagnosing periodontal patients early on and to continue to follow the progress of the disease with minimal intervention and could become part of the routine dental visits. It should be noted, however, that the samples collected during a routine visit should be tested immediately after collection since storage of samples at −20°C for 2–3 months has been shown to alter the protein profiles (Preiano et al., 2016).

Metabolomics

The high throughput analysis and biological understanding of how metabolites contribute to disease processes through metabolomics is an emerging field. We can appreciate the value of sequencing technologies in study of health and disease, however if one was interested in developing a bed or chair side point of care diagnostic device or test, metabolites would be a desirable analyte for detection.

Are There Bacterial Contributions to the Salivary Metabolome?

The salivary metabolomic studies are rapidly advancing and in this regard, it is prudent to understand the host-bacterial contributions when saliva is used as the diagnostic fluid. NMR has been utilized specifically for this purpose. For example, saliva is a diagnostic fluid that has been used in the metabolic profiling of various oral diseases such as caries. In this regard Gardner et al., have shown that there is significant contribution from the oral microbiota in unstimulated whole mouth saliva (Gardner et al., 2019). Specifically, when saliva samples of healthy volunteers were analyzed, using ^{1}H-NMR, CFU enumeration, and principal component analysis, it was shown that whole saliva metabolites were positively correlated with bacterial load suggesting that the metabolite composition of whole saliva is more reflective of the oral microbiota than the underlying host metabolism, which was determined from plasma levels and parotid saliva. The whole saliva contained abundant short-chain fatty acids (acetate, propionate, butyrate, and formate) compared to parotic saliva or plasma. The authors conclude that whole saliva might be particularly useful in conjunction with NMR analysis to diagnose conditions reflective of dysbiosis. A comparative study of subjects with dental diseases might be useful for future dysbiosis studies (Gardner et al., 2019).

Metabolomics and Periodontitis

Like mass spectrometry, ^{1}H-NMR has increasingly becoming a tool to study the metabolites associated with disease conditions. In a study that analyzed the NMR profiles of healthy control ($n = 52$) and post-treatment chronic periodontitis patients ($n = 62$) using un-stimulated saliva, 100 metabolites were characterized (Singh et al., 2019). Distinctive differences in the spectral data were subjected to multivariate analysis, which showed that there is an elevation in the concentration of statistically discriminant metabolites between control and diseased patient profiles. Among the 100 metabolites studied, 20 new metabolites indicate a bacterial population shift along with change in homeostasis, which might disturb the biofilm composition. Decreasing levels of N-acetylglucosamine along with pyruvate, glutamate, and ethane sulfonate support a shift from homeostatic to anaerobic conditions, a defining characteristic of the severity of chronic periodontitis (Aimetti et al., 2012). The Singh and Aimetti studies suggest that decreased levels of pyruvate and N-acetylglucosamine may be a signature in chronic periodontitis (Aimetti et al., 2012; Singh et al., 2019).

CONCLUSION

Here in this review, we have presented how dysbiosis that occurs within host domains, and in particular the oral cavity, can affect disease. We have also outlined the current "omic" technologies that will allow researchers to examine the system as a whole in the future. Our particular emphasis has been on the role that commensals and pathobionts play in their interaction with the immune status of the host. It is apparent when considering the progress made in characterizing the oral microbiome and the oral immune environment that we are poised to begin to synthesize accurate models of the relative contributions of these components to disease.

While we have presented historical and new/advanced technologies that have been and will continue to be used to diagnose and assess the two most common dental diseases, caries and periodontal diseases we feel compelled to provide several cautionary notes worthy of comment. First, no technology can advance our understanding of disease until we have a unified, accepted, and clearly defined definition of health and disease. No matter how sophisticated the technology, poor and inconsistent definitions of disease will continue to lead to confusion rather than clarity. This is especially true since both microbial and host dysbiosis is so critical to shifts from health to disease in the oral cavity. Second, whatever technology we choose to utilize, we should make every effort to include data that is coordinated, comprehensive, and includes microbiology, host responsiveness, and disease progression or resolution. Since it is becoming clearer that oral disease has a relationship to overall health and well-being we need to extend our concerns related to how local oral

diseases can effect overall general well-being. Third, since the diseases we study go through spurts of activity that vary from time to time, longitudinal rather than cross-sectional studies are preferred.

In this age of advanced technology there are several issues that need to be addressed if we are to advance the field of infectious diseases. In our field key issues relate to sampling, sample storage, analyte extraction and processing, data analysis, presentation and interpretation of results. As reports are published, the methods section has to be explicit in the description of the processes if we are to compare data and make meaningful conclusions among these large data sets. As an example to consider the level of detail needed the following must be addressed: when samples are taken is there a separation of supragingival and subgingival plaque, is the plaque collected by curette or paper point or some other device, are samples pooled or kept in independent vials, is there mention of the time of plaque collection, is it before, during or after disease has occurred? For analyte extraction what are the methods, has bead beating, sonication, biochemical methods been performed and for what time period, has the cell wall has been breached? Has the volume of analyte been standardized? With what technology platform are the samples analyzed? What pre and post-data analysis programs are used? Is data generated compared to a standardized database and if so which? What data analysis and statistical analysis is being conducted? Is Principal Component Analysis used as an initial determinant? How much is data tied to alpha and beta diversity, Shannon Diversity? Network Analysis of what kind? Linear Discriminant Analysis? These are a cross-section of questions that need to be described in each publication to reassure standardization of methodologies such that comparison of data is possible.

Finally, while we recognize that these criteria are demanding and difficult to accomplish our hope is that this review and the technologies presented herewith will inspire new ways of tackling persistent uncertainties. These obstinate questions have left us with huge gaps in our knowledge base in our efforts to both diagnose and treat oral diseases. While oral biologists do not stand alone in this dilemma perhaps this overview will be of some assistance in efforts to advance our understanding in the future.

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CC, NR, VT, and DF contributed equally to conceptualizing, writing, and editing the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Abel, B., Thieblemont, N., Quesniaux, V. J., Brown, N., Mpagi, J., Miyake, K., et al. (2002). Toll-like receptor 4 expression is required to control chronic *Mycobacterium tuberculosis* infection in mice. *J. Immunol.* 169, 3155–3162. doi: 10.4049/jimmunol.169.6.3155

Abelson, D. C., and Mandel, I. D. (1981). The effect of saliva on plaque pH *in vivo*. *J. Dent. Res.* 60, 1634–1638. doi: 10.1177/00220345810600090101

Abt, M. C., and Pamer, E. G. (2014). Commensal bacteria mediated defenses against pathogens. *Curr. Opin. Immunol.* 29, 16–22. doi: 10.1016/j.coi.2014.03.003

Abusleme, L., Dupuy, A. K., Dutzan, N., Silva, N., Burleson, J. A., Strausbaugh, L. D., et al. (2013). The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J.* 7, 1016–1025. doi: 10.1038/ismej.2012.174

Adamus, T., and Kortylewski, M. (2018). The revival of CpG oligonucleotide-based cancer immunotherapies. *Contemp. Oncol.* 22, 56–60. doi: 10.5114/wo.2018.73887

Ai, D., Huang, R., Wen, J., Li, C., Zhu, J., and Xia, L. C. (2017). Integrated metagenomic data analysis demonstrates that a loss of diversity in oral microbiota is associated with periodontitis. *BMC Genomics* 18:1041. doi: 10.1186/s12864-016-3254-5

Aimetti, M., Cacciatore, S., Graziano, A., and Tenori, L. (2012). Metabonomic analysis of saliva reveals generalized chronic periodontitis signature. *Metabolomics* 8, 465–474. doi: 10.1007/s11306-011-0331-2

Alm, J. S., Swartz, J., Björkstén, B., Engstrand, L., Engström, J., Kühn, I., et al. (2002). An anthroposophic lifestyle and intestinal microflora in infancy. *Pediatr. Allergy Immunol.* 13, 402–411. doi: 10.1034/j.1399-3038.2002.01062.x

Al-Sabbagh, M., Alladah, A., Lin, Y., Kryscio, R. J., Thomas, M. V., Ebersole, J. L., et al. (2012). Bone remodeling-associated salivary biomarker MIP-1alpha distinguishes periodontal disease from health. *J. Periodont. Res.* 47, 389–395. doi: 10.1111/j.1600-0765.2011.01445.x

Antezack, A., Chaudet, H., Tissot-Dupont, H., Brouqui, P., and Monnet-Corti, V. (2020). Rapid diagnosis of periodontitis, a feasibility study using MALDI-TOF mass spectrometry. *PLoS ONE* 15:e0230334. doi: 10.1371/journal.pone.0230334

Araujo-Pires, A. C., Vieira, A. E., Francisconi, C. F., Biguetti, C. C., Glowacki, A., Yoshizawa, S., et al. (2015). IL-4/CCL22/CCR4 axis controls regulatory T-cell migration that suppresses inflammatory bone loss in murine experimental periodontitis. *J. Bone Miner. Res.* 30, 412–422. doi: 10.1002/jbmr.2376

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., et al. (2011). Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science* 331, 337–341. doi: 10.1126/science.1198469

Baggio, G., Donazzan, S., Monti, D., Mari, D., Martini, S., Gabelli, C., et al. (1998). Lipoprotein(a) and lipoprotein profile in healthy centenarians: a reappraisal of vascular risk factors. *FASEB J.* 12, 433–437. doi: 10.1096/fasebj.12.6.433

Beckwith, T. D., Simonton, F. V., and Williams, A. (1925). A histologic study of the gum in Pyorrhea*, **Aided by grants from the Carnegie Corporation., the American Dental Association and the Associated Radiograph Laboratories.*From the Department of Bacteriology, University of California, Berkeley, University of California Dental School, San Francisco, and California Stomatological Research Group. *J. Am. Dental Assoc.* 12, 129–153. doi: 10.14219/jada.archive.1925.0045

Beckwith, T. D., Williams, A., and Rose, E. T. (1929). The role of bacteria in pyorrhea. *Med. J. Rec.* 129, 333–336.

Benitez-Paez, A., Belda-Ferre, P., Simon-Soro, A., and Mira, A. (2014). Microbiota diversity and gene expression dynamics in human oral biofilms. *BMC Genomics* 15:311. doi: 10.1186/1471-2164-15-311

Beringer, A., and Miossec, P. (2019). Systemic effects of IL-17 in inflammatory arthritis. *Nat. Rev. Rheumatol.* 15, 491–501. doi: 10.1038/s41584-019-0243-5

Berkowitz, R. J., and Jones, P. (1985). Mouth-to-mouth transmission of the bacterium *Streptococcus mutans* between mother and child. *Arch. Oral Biol.* 30, 377–379. doi: 10.1016/0003-9969(85)90014-7

Beutler, B. (2013). Bruce Beutler: the persistent prospector. *Interview by Ruth Williams. Circ. Res.* 112, 751–754. doi: 10.1161/CIRCRESAHA.113.301093

Bhavsar, I., Miller, C., and Al-Sabbagh, M. (2015). "Macrophage inflammatory protein-1 alpha (MIP-1 alpha)/CCL3: as a biomarker," in *General Methods in Biomarker Research and their Applications, Vol. 1,* eds V. Preedy and V. Patel (Dordrecht: Springer), 223–249. doi: 10.1007/978-94-007-7696-8_27

Billings, F. (1912). Chronic focal infections and their etiologic relations to arthritis and nephritis. *Archiv. Int. Med.* 9, 484–498. doi: 10.1001/archinte.1912.00060160087007

Bowdish, D. M., Sakamoto, K., Kim, M. J., Kroos, M., Mukhopadhyay, S., Leifer, C. A., et al. (2009). MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and *Mycobacterium tuberculosis*. *PLoS Pathog.* 5:e1000474. doi: 10.1371/journal.ppat.1000474

Boyle, W. J., Simonet, W. S., and Lacey, D. L. (2003). Osteoclast differentiation and activation. *Nature* 423, 337–342. doi: 10.1038/nature01658

Brown, K. A., Khanafer, N., Daneman, N., and Fisman, D. N. (2013). Meta-analysis of antibiotics and the risk of community-associated *Clostridium difficile* infection. *Antimicrob. Agents Chemother.* 57, 2326–2332. doi: 10.1128/AAC.02176-12

Bryant, J., and Thistle, J. (2020). "Anatomy, colostrum," in *StatPearls* (Treasure Island, FL: StatPearls Publishing.

Buffie, C. G., Jarchum, I., Equinda, M., Lipuma, L., Gobourne, A., Viale, A., et al. (2012). Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to *Clostridium difficile*-induced colitis. *Infect. Immun.* 80, 62–73. doi: 10.1128/IAI.05496-11

Buzalaf, M. A. R., Ortiz, A. C., Carvalho, T. S., Fideles, S. O. M., Araujo, T. T., et al. (2020). Saliva as a diagnostic tool for dental caries, periodontal disease and cancer: is there a need for more biomarkers? *Expert Rev. Mol. Diagn.* 20, 543–555. doi: 10.1080/14737159.2020.1743686

Canaday, D. H., Amponsah, N. A., Jones, L., Tisch, D. J., Hornick, T. R., and Ramachandra, L. (2010). Influenza-induced production of interferon-alpha is defective in geriatric individuals. *J. Clin. Immunol.* 30, 373–383. doi: 10.1007/s10875-010-9374-9

Cardoso, C. R., Garlet, G. P., Crippa, G. E., Rosa, A. L., Júnior, W. M., Rossi, M. A., et al. (2009). Evidence of the presence of T helper type 17 cells in chronic lesions of human periodontal disease. *Oral Microbiol. Immunol.* 24, 1–6. doi: 10.1111/j.1399-302X.2008.00463.x

Casadevall, A., and Pirofski, L. A. (1999). Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infect. Immun.* 67, 3703–3713. doi: 10.1128/IAI.67.8.3703-3713.1999

Casadevall, A., and Pirofski, L. A. (2003). The damage-response framework of microbial pathogenesis. *Nat. Rev. Microbiol.* 1, 17–24. doi: 10.1038/nrmicro732

Casadevall, A., and Pirofski, L. A. (2015). What is a host? *Incorporating the microbiota into the damage-response framework. Infect. Immun.* 83, 2–7. doi: 10.1128/IAI.02627-14

Casadevall, A., and Pirofski, L. A. (2018). What Is a Host? *Attributes of individual susceptibility. Infect. Immun.* 86:e00636-17. doi: 10.1128/IAI.00636-17

Cecil, R. L. (1929). *A Text-book of Medicine: by American Authors*. Philadelphia: Saunders.

Cecil, R. L., and Angevine, D. M. (1938). Clinical and experimental observations of focal infection, with an analysis of 200 cases of rheumatoid arthritis. *Ann. Intern. Med.* 12, 577–584. doi: 10.7326/0003-4819-12-5-577

Chen, C., Hemme, C., Beleno, J., Shi, Z. J., Ning, D., Qin, Y., et al. (2018). Oral microbiota of periodontal health and disease and their changes after nonsurgical periodontal therapy. *ISME J.* 12, 1210–1224. doi: 10.1038/s41396-017-0037-1

Chmiela, M., and Gonciarz, W. (2017). Molecular mimicry in *Helicobacter pylori* infections. *World J. Gastroenterol.* 23, 3964–3977. doi: 10.3748/wjg.v23.i22.3964

Cho, I., and Blaser, M. J. (2012). The human microbiome: at the interface of health and disease. *Nat. Rev. Genet.* 13, 260–270. doi: 10.1038/nrg3182

Chung, P. L., Zhou, S., Eslami, B., Shen, L., Leboff, M. S., and Glowacki, J. (2014). Effect of age on regulation of human osteoclast differentiation. *J. Cell. Biochem.* 115, 1412–1419. doi: 10.1002/jcb.24792

Clarke, J. K. (1924). On the bacterial factor in the ætiology of dental caries. *Br. J. Exp. Pathol.* 5, 141–147.

Clarke, T. B., Davis, K. M., Lysenko, E. S., Zhou, A. Y., Yu, Y., and Weiser, J. N. (2010). Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* 16, 228–231. doi: 10.1038/nm.2087

Colombo, A. P., Boches, S. K., Cotton, S. L., Goodson, J. M., Kent, R., Haffajee, A. D., et al. (2009). Comparisons of subgingival microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. *J. Periodontol.* 80, 1421–1432. doi: 10.1902/jop.2009.090185

Colyer, S. (1902). Oral sepsis: and some of its effects. *Dent. Rec.* 20, 200–206.

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J., and Relman, D. A. (2012). The application of ecological theory toward an understanding of the human microbiome. *Science* 336, 1255–1262. doi: 10.1126/science.1224203

Cox, L. M., Yamanishi, S., Sohn, J., Alekseyenko, A. V., Leung, J. M., Cho, I., et al. (2014). Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. *Cell* 158, 705–721. doi: 10.1016/j.cell.2014.05.052

Cugini, C., Klepac-Ceraj, V., Rackaityte, E., Riggs, J. E., and Davey, M. E. (2013). *Porphyromonas gingivalis*: keeping the pathos out of the biont. *J. Oral Microbiol.* 5:1. doi: 10.3402/jom.v5i0.19804

Cullen, C. M., Aneja, K. K., Beyhan, S., Cho, C. E., Woloszynek, S., Convertino, M., et al. (2020). Emerging priorities for microbiome research. *Front. Microbiol.* 11:136. doi: 10.3389/fmicb.2020.00136

Curtis, M. A., Diaz, P. I., and Van Dyke, T. E. (2020). The role of the microbiota in periodontal disease. *Periodontol. 2000* 83, 14–25. doi: 10.1111/prd.12296

da Motta, R. J., Tirapelli, C., Juns Da Silva, R., Villafuerte, K. R., Almeida, L. Y., Ribeiro-Silva, A., et al. (2016). Immature, but not mature, dendritic cells are more often present in aggressive periodontitis than chronic periodontitis: an immunohistochemical study. *J. Periodontol.* 87, 1499–1507. doi: 10.1902/jop.2016.150729

D'Aiuto, F., Parkar, M., Andreou, G., Suvan, J., Brett, P. M., Ready, D., et al. (2004). Periodontitis and systemic inflammation: control of the local infection is associated with a reduction in serum inflammatory markers. *J. Dent. Res.* 83, 156–160. doi: 10.1177/154405910408300214

Dashper, S. G., and Reynolds, E. C. (1996). Lactic acid excretion by *Streptococcus mutans*. *Microbiology* 142, 33–39. doi: 10.1099/13500872-142-1-33

Dawes, C. (2003). What is the critical pH and why does a tooth dissolve in acid? *J. Can. Dent. Assoc.* 69, 722–724.

De Lorenzo, G., Ferrari, S., Cervone, F., and Okun, E. (2018). Extracellular DAMPs in plants and mammals: immunity, tissue damage and repair. *Trends Immunol.* 39, 937–950. doi: 10.1016/j.it.2018.09.006

Delima, A. J., and Van Dyke, T. E. (2003). Origin and function of the cellular components in gingival crevice fluid. *Periodontol. 2000* 31, 55–76. doi: 10.1034/j.1600-0757.2003.03105.x

Dethlefsen, L., Huse, S., Sogin, M. L., and Relman, D. A. (2008). The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol.* 6:e280. doi: 10.1371/journal.pbio.0060280

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W. H., et al. (2010). The human oral microbiome. *J. Bacteriol.* 192, 5002–5017. doi: 10.1128/JB.00542-10

Diaz, P. I., Hoare, A., and Hong, B. Y. (2016). Subgingival microbiome shifts and community dynamics in periodontal diseases. *J. Calif. Dent. Assoc.* 44, 421–435.

Dominguez-Bello, M. G., Godoy-Vitorino, F., Knight, R., and Blaser, M. J. (2019). Role of the microbiome in human development. *Gut* 68, 1108–1114. doi: 10.1136/gutjnl-2018-317503

Dorey, R. B., Theodosiou, A. A., Read, R. C., and Jones, C. E. (2019). The nonpathogenic commensal *Neisseria*: friends and foes in infectious disease. *Curr. Opin. Infect. Dis.* 32, 490–496. doi: 10.1097/QCO.0000000000000585

Doxey, A. C., and McConkey, B. J. (2013). Prediction of molecular mimicry candidates in human pathogenic bacteria. *Virulence* 4, 453–466. doi: 10.4161/viru.25180

Duguid, R. (1985). *In-vitro* acid production by the oral bacterium *Streptococcus mutans* 10449 in various concentrations of glucose, fructose and sucrose. *Arch. Oral Biol.* 30, 319–324. doi: 10.1016/0003-9969(85)90004-4

Duran-Pinedo, A. E., Baker, V. D., and Frias-Lopez, J. (2014a). The periodontal pathogen *Porphyromonas gingivalis* induces expression of transposases and cell

death of *Streptococcus mitis* in a biofilm model. *Infect. Immun.* 82, 3374–3382. doi: 10.1128/IAI.01976-14

Duran-Pinedo, A. E., Chen, T., Teles, R., Starr, J. R., Wang, X., Krishnan, K., et al. (2014b). Community-wide transcriptome of the oral microbiome in subjects with and without periodontitis. *ISME J.* 8, 1659–1672. doi: 10.1038/ismej.2014.23

Dutzan, N., Kajikawa, T., Abusleme, L., Greenwell-Wild, T., Zuazo, C. E., Ikeuchi, T., et al. (2018). A dysbiotic microbiome triggers TH17 cells to mediate oral mucosal immunopathology in mice and humans. *Sci. Transl. Med.* 10:eaat0797. doi: 10.1126/scitranslmed.aat0797

Dutzan, N., Konkel, J. E., Greenwell-Wild, T., and Moutsopoulos, N. M. (2016). Characterization of the human immune cell network at the gingival barrier. *Mucosal Immunol.* 9, 1163–1172. doi: 10.1038/mi.2015.136

Ebersole, J. L., Dawson, D. 3rd, Emecen-Huja, P., Nagarajan, R., Howard, K., Grady, M. E., Thompson, K., et al. (2017). The periodontal war: microbes and immunity. *Periodontol. 2000* 75, 52–115. doi: 10.1111/prd.12222

Ebersole, J. L., Graves, C. L., Gonzalez, O. A., Dawson, D. III, Morford, L. A., Huja, P. E., et al. (2016). Aging, inflammation, immunity and periodontal disease. *Periodontol. 2000* 72, 54–75. doi: 10.1111/prd.12135

Echchannaoui, H., Frei, K., Schnell, C., Leib, S. L., Zimmerli, W., and Landmann, R. (2002). Toll-like receptor 2-deficient mice are highly susceptible to *Streptococcus pneumoniae* meningitis because of reduced bacterial clearing and enhanced inflammation. *J. Infect. Dis.* 186, 798–806. doi: 10.1086/342845

Elde, N. C., and Malik, H. S. (2009). The evolutionary conundrum of pathogen mimicry. *Nat. Rev. Microbiol.* 7, 787–797. doi: 10.1038/nrmicro2222

Ellekilde, M., Selfjord, E., Larsen, C. S., Jakesevic, M., Rune, I., Tranberg, B., et al. (2014). Transfer of gut microbiota from lean and obese mice to antibiotic-treated mice. *Sci. Rep.* 4:5922. doi: 10.1038/srep05922

Englander, H. R., and Keyes, P. H. (1964). Failure to induce dental caries in hamsters by transfer of plaque and carious debris from humans. *J. Dent. Res.* 43:626. doi: 10.1177/00220345640430041701

Esberg, A., Barone, A., Eriksson, L., Lif Holgerson, P., Teneberg, S., and Johansson, I. (2020). *Corynebacterium matruchotii* demography and adhesion determinants in the oral cavity of healthy individuals. *Microorganisms* 8:1780. doi: 10.3390/microorganisms8111780

Falk, P. G., Hooper, L. V., Midtvedt, T., and Gordon, J. I. (1998). Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol. Mol. Biol. Rev.* 62, 1157–1170. doi: 10.1128/MMBR.62.4.1157-1170.1998

Feng, X., and McDonald, J. M. (2011). Disorders of bone remodeling. *Annu. Rev. Pathol.* 6, 121–145. doi: 10.1146/annurev-pathol-011110-130203

Fetissov, S. O., Hamze Sinno, M., Coëffier, M., Bole-Feysot, C., Ducrotté, P., Hökfelt, T., et al. (2008). Autoantibodies against appetite-regulating peptide hormones and neuropeptides: putative modulation by gut microflora. *Nutrition* 24, 348–359. doi: 10.1016/j.nut.2007.12.006

Fine, D. H. (1995). Chemical agents to prevent and regulate plaque development. *Periodontol. 2000* 8, 87–107. doi: 10.1111/j.1600-0757.1995.tb00047.x

Fine, D. H. (2006). Dr. Theodor Rosebury: grandfather of modern oral microbiology. *J. Dent. Res.* 85, 990–995. doi: 10.1177/154405910608501103

Fine, D. H., Korik, I., Furgang, D., Myers, R., Olshan, A., Barnett, M. L., et al. (1996). Assessing pre-procedural subgingival irrigation and rinsing with an antiseptic mouthrinse to reduce bacteremia. *JADA* 127, 641–646. doi: 10.14219/jada.archive.1996.0276

Fine, D. H., Markowitz, K., Fairlie, K., Tischio-Bereski, D., Ferrandiz, J., Godboley, D., et al. (2014). Macrophage inflammatory protein-1alpha shows predictive value as a risk marker for subjects and sites vulnerable to bone loss in a longitudinal model of aggressive periodontitis. *PLoS ONE* 9:e98541. doi: 10.1371/journal.pone.0098541

Fine, D. H., Markowitz, K., Fairlie, K., Tischio-Bereski, D., Ferrendiz, J., Furgang, D., et al. (2013). A consortium of *Aggregatibacter actinomycetemcomitans*, *Streptococcus parasanguinis*, and *Filifactor alocis* is present in sites prior to bone loss in a longitudinal study of localized aggressive periodontitis. *J. Clin. Microbiol.* 51, 2850–2861. doi: 10.1128/JCM.00729-13

Fine, D. H., Markowitz, K., Furgang, D., Fairlie, K., Ferrandiz, J., Nasri, C., et al. (2009). Macrophage inflammatory protein-1alpha: a salivary biomarker of bone loss in a longitudinal cohort study of children at risk for aggressive periodontal disease? *J. Periodontol.* 80, 106–113. doi: 10.1902/jop.2009.080296

Fitzgerald, R. J., and Keyes, P. H. (1960). Demonstration of the etiologic role of streptococci in experimental caries in the hamster. *J. Am. Dent. Assoc.* 61, 9–19. doi: 10.14219/jada.archive.1960.0138

Fitzgerald, R. J., and Keyes, P. H. (1963). Ecologic factors in dental caries. the fate of antibiotic-resistant cariogenic streptococci in hamsters. *Am. J. Pathol.* 42, 759–772.

Foster, K. R., Schluter, J., Coyte, K. Z., and Rakoff-Nahoum, S. (2017). The evolution of the host microbiome as an ecosystem on a leash. *Nature* 548, 43–51. doi: 10.1038/nature23292

Fulop, T., Witkowski, J. M., Olivieri, F., and Larbi, A. (2018). The integration of inflammaging in age-related diseases. *Semin. Immunol.* 40, 17–35. doi: 10.1016/j.smim.2018.09.003

Funaki, Y., Hasegawa, Y., Okazaki, R., Yamasaki, A., Sueda, Y., Yamamoto, A., et al. (2018). Resolvin E1 inhibits osteoclastogenesis and bone resorption by suppressing IL-17-induced RANKL expression in osteoblasts and RANKL-induced osteoclast differentiation. *Yonago Acta Med.* 61, 8–18. doi: 10.33160/yam.2018.03.002

Gardner, A., Parkes, H. G., So, P.-W., and Carpenter, G. H. (2019). Determining bacterial and host contributions to the human salivary metabolome. *J. Oral Microbiol.* 11:1617014. doi: 10.1080/20002297.2019.1617014

Gilleron, M., Quesniaux, V. F., and Puzo, G. (2003). Acylation state of the phosphatidylinositol hexamannosides from *Mycobacterium bovis* bacillus Calmette Guerin and mycobacterium tuberculosis H37Rv and its implication in Toll-like receptor response. *J. Biol. Chem.* 278, 29880–29889. doi: 10.1074/jbc.M303446200

Girardin, S. E., Boneca, I. G., Carneiro, L. A., Antignac, A., Jéhanno, M., Viala, J., et al. (2003). Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300, 1584–1587. doi: 10.1126/science.1084677

Glowacki, A. J., Yoshizawa, S., Jhunjhunwala, S., Vieira, A. E., Garlet, G. P., Sfeir, C., et al. (2013). Prevention of inflammation-mediated bone loss in murine and canine periodontal disease via recruitment of regulatory lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 110, 18525–18530. doi: 10.1073/pnas.1302829110

Gordon, S. (2008). Elie Metchnikoff: father of natural immunity. *Eur. J. Immunol.* 38, 3257–3264. doi: 10.1002/eji.200838855

Gordon, S. (2016). Elie Metchnikoff, the Man and the Myth. *J. Innate Immun.* 8, 223–227. doi: 10.1159/000443331

Griffen, A. L., Beall, C. J., Campbell, J. H., Firestone, N. D., Kumar, P. S., Yang, Z. K., et al. (2012). Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME J.* 6, 1176–1185. doi: 10.1038/ismej.2011.191

Haffajee, A. D., Patel, M., and Socransky, S. S. (2008). Microbiological changes associated with four different periodontal therapies for the treatment of chronic periodontitis. *Oral Microbiol. Immunol.* 23, 148–157. doi: 10.1111/j.1399-302X.2007.00403.x

Hagenfeld, D., Koch, R., Junemann, S., Prior, K., Harks, I., Eickholz, P., et al. (2018). Do we treat our patients or rather periodontal microbes with adjunctive antibiotics in periodontal therapy? A 16S rDNA microbial community analysis. *PLoS ONE* 13:e0195534. doi: 10.1371/journal.pone.0195534

Hajishengallis, G., and Moutsopoulos, N. M. (2014). Etiology of leukocyte adhesion deficiency-associated periodontitis revisited: not a raging infection but a raging inflammatory response. *Expert Rev. Clin. Immunol.* 10, 973–975. doi: 10.1586/1744666X.2014.929944

Hajishengallis, G., and Moutsopoulos, N. M. (2016). Role of bacteria in leukocyte adhesion deficiency-associated periodontitis. *Microb. Pathog.* 94, 21–26. doi: 10.1016/j.micpath.2015.09.003

Hanna, S., and Etzioni, A. (2012). Leukocyte adhesion deficiencies. *Ann. N. Y. Acad. Sci.* 1250, 50–55. doi: 10.1111/j.1749-6632.2011.06389.x

Hao, J., Liu, R., Piao, W., Zhou, Q., Vollmer, T. L., Campagnolo, D. I., et al. (2010). Central nervous system (CNS)-resident natural killer cells suppress Th17 responses and CNS autoimmune pathology. *J. Exp. Med.* 207, 1907–1921. doi: 10.1084/jem.20092749

Harding, C. V., and Boom, W. H. (2010). Regulation of antigen presentation by *Mycobacterium tuberculosis*: a role for Toll-like receptors. *Nat. Rev. Microbiol.* 8, 296–307. doi: 10.1038/nrmicro2321

Hartenbach, F., Velasquez, E., Nogueira, F. C. S., Domont, G. B., Ferreira, E., and Colombo, A. P. V. (2020). Proteomic analysis of whole saliva in chronic periodontitis. *J. Proteomics* 213:103602. doi: 10.1016/j.jprot.2019.103602

Hausmann, A., Böck, D., Geiser, P., Berthold, D. L., Fattinger, S. A., Furter, M., et al. (2020). Intestinal epithelial NAIP/NLRC4 restricts systemic dissemination of the adapted pathogen *Salmonella Typhimurium* due to site-specific bacterial PAMP expression. *Mucosal Immunol.* 13, 530–544. doi: 10.1038/s41385-019-0247-0

He, J., Fu, W., Zhao, S., Zhang, C., Sun, T., and Jiang, T. (2019). Lack of MSMEG_6281, a peptidoglycan amidase, affects cell wall integrity and virulence of *Mycobacterium smegmatis*. *Microb. Pathog.* 128, 405–413. doi: 10.1016/j.micpath.2019.01.013

Hojo, K., Nagaoka, S., Ohshima, T., and Maeda, N. (2009). Bacterial interactions in dental biofilm development. *J. Dent. Res.* 88, 982–990. doi: 10.1177/0022034509346811

Hook, J. S., Cao, M., Weng, K., Kinnare, N., and Moreland, J. G. (2020). *Mycobacterium tuberculosis* lipoarabinomannan activates human neutrophils via a TLR2/1 mechanism distinct from Pam(3)CSK(4). *J. Immunol.* 204, 671–681. doi: 10.4049/jimmunol.1900919

Hooks, K. B., and O'Malley, M. A. (2017). Dysbiosis and its discontents. *MBio* 8:e01492-17. doi: 10.1128/mBio.01492-17

Hsiao, E. Y., Mcbride, S. W., Hsien, S., Sharon, G., Hyde, E. R., Mccue, T., et al. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 155, 1451–1463. doi: 10.1016/j.cell.2013.11.024

Huang, C. B., Alimova, Y., and Ebersole, J. L. (2016). Macrophage polarization in response to oral commensals and pathogens. *Pathog. Dis.* 74:ftw011. doi: 10.1093/femspd/ftw011

Human Microbiome Project, C. (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234

Hunter, W. (1900). Oral sepsis as a cause of disease. *Br. Med. J.* 2, 215–216. doi: 10.1136/bmj.2.2065.215

Hunter, W. (1911). *The Role of Sepsis and Antisepsis in Medicine and the Importance of Oral Sepsis as Its Chief Cause*. The Dental Register LXV, 579.

Jakubovics, N. S., Gill, S. R., Iobst, S. E., Vickerman, M. M., and Kolenbrander, P. E. (2008a). Regulation of gene expression in a mixed-genus community: stabilized arginine biosynthesis in *Streptococcus gordonii* by coaggregation with *Actinomyces naeslundii*. *J. Bacteriol.* 190, 3646–3657. doi: 10.1128/JB.00088-08

Jakubovics, N. S., Gill, S. R., Vickerman, M. M., and Kolenbrander, P. E. (2008b). Role of hydrogen peroxide in competition and cooperation between *Streptococcus gordonii* and *Actinomyces naeslundii*. *FEMS Microbiol. Ecol.* 66, 637–644. doi: 10.1111/j.1574-6941.2008.00585.x

James, J. (1994). Van Leeuwenhoek's discoveries of 1677–1678: a look too far. *Micron* 25, 1–4. doi: 10.1016/0968-4328(94)90050-7

Janeway, C. A. Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* 54(Pt 1), 1–13. doi: 10.1101/SQB.1989.054.01.003

Jorth, P., Turner, K. H., Gumus, P., Nizam, N., Buduneli, N., and Whiteley, M. (2014). Metatranscriptomics of the human oral microbiome during health and disease. *MBio* 5:e01012-14. doi: 10.1128/mBio.01012-14

Jotwani, R., Palucka, A. K., Al-Quotub, M., Nouri-Shirazi, M., Kim, J., Bell, D., et al. (2001). Mature dendritic cells infiltrate the T cell-rich region of oral mucosa in chronic periodontitis: *in situ*, *in vivo*, and *in vitro* studies. *J. Immunol.* 167, 4693–4700. doi: 10.4049/jimmunol.167.8.4693

Kabat, E. A. (1983). Getting started 50 years ago–experiences, perspectives, and problems of the first 21 years. *Annu. Rev. Immunol.* 1, 1–32. doi: 10.1146/annurev.iy.01.040183.000245

Kamboj, A. K., Cotter, T. G., and Oxentenko, A. S. (2017). *Helicobacter pylori*: the past, present, and future in management. *Mayo Clin. Proc.* 92, 599–604. doi: 10.1016/j.mayocp.2016.11.017

Karki, R., Lee, E., Place, D., Samir, P., Mavuluri, J., Sharma, B. R., et al. (2018). IRF8 regulates transcription of naips for NLRC4 inflammasome activation. *Cell* 173, 920.e13–933.e13. doi: 10.1016/j.cell.2018.02.055

Kaufmann, S. H. E. (2017). Remembering Emil von Behring: from tetanus treatment to antibody cooperation with phagocytes. *MBio* 8:e00117-17. doi: 10.1128/mBio.00117-17

Kay, J. G., Kramer, J. M., and Visser, M. B. (2019). Danger signals in oral cavity-related diseases. *J. Leukoc. Biol.* 106, 193–200. doi: 10.1002/JLB.4MIR1118-439R

Kellam, P., and Weiss, R. A. (2006). Infectogenomics: insights from the host genome into infectious diseases. *Cell* 124, 695–697. doi: 10.1016/j.cell.2006.02.003

Kennedy, E. A., King, K. Y., and Baldridge, M. T. (2018). Mouse microbiota models: comparing germ-free mice and antibiotics treatment as tools for modifying gut bacteria. *Front. Physiol.* 9:1534. doi: 10.3389/fphys.2018.01534

Keyes, P. H., and Fitzgerald, R. J. (1962). Dental caries in the Syrian hamster. IX. *Arch. Oral Biol.* 7, 267–277. doi: 10.1016/0003-9969(62)90017-1

Kianoush, N., Adler, C. J., Nguyen, K. A., Browne, G. V., Simonian, M., and Hunter, N. (2014). Bacterial profile of dentine caries and the impact of pH on bacterial population diversity. *PLoS ONE* 9:e92940. doi: 10.1371/journal.pone.0092940

Kim, D., Yoo, S. A., and Kim, W. U. (2016). Gut microbiota in autoimmunity: potential for clinical applications. *Arch. Pharm. Res.* 39, 1565–1576. doi: 10.1007/s12272-016-0796-7

Kim, W. J., Higashi, D., Goytia, M., Rendon, M. A., Pilligua-Lucas, M., Bronnimann, M., et al. (2019). Commensal *Neisseria* kill *Neisseria gonorrhoeae* through a DNA-dependent mechanism. *Cell Host Microbe* 26, 228.e8–239.e8. doi: 10.1016/j.chom.2019.07.003

Kiyono, H., and Azegami, T. (2015). The mucosal immune system: from dentistry to vaccine development. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* 91, 423–439. doi: 10.2183/pjab.91.423

Kiyono, H., and Fukuyama, S. (2004). NALT- versus Peyer's-patch-mediated mucosal immunity. *Nat. Rev. Immunol.* 4, 699–710. doi: 10.1038/nri1439

Kobayashi, S. D., Malachowa, N., and Deleo, F. R. (2018). Neutrophils and bacterial immune evasion. *J. Innate Immun.* 10, 432–441. doi: 10.1159/000487756

Koch, R. (1893). Ueber den augenblicklichen Stand der bakteriologischen Choleradiagnose. *Zeitschrift für Hygiene und Infektionskrankheiten* 14, 319–338. doi: 10.1007/BF02284324

Kofoed, E. M., and Vance, R. E. (2011). Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* 477, 592–595. doi: 10.1038/nature10394

Kolenbrander, P. E. (2000). Oral microbial communities: biofilms, interactions, and genetic systems. *Annu. Rev. Microbiol.* 54, 413–437. doi: 10.1146/annurev.micro.54.1.413

Kolenbrander, P. E., Andersen, R. N., Blehert, D. S., Egland, P. G., Foster, J. S., and Palmer, R. J. Jr. (2002). Communication among oral bacteria. *Microbiol. Mol. Biol. Rev.* 66, 486–505. doi: 10.1128/MMBR.66.3.486-505.2002

Kolenbrander, P. E., Inouye, Y., and Holdeman, L. V. (1983). New *Actinomyces* and *Streptococcus* coaggregation groups among human oral isolates from the same site. *Infect. Immun.* 41, 501–506. doi: 10.1128/IAI.41.2.501-506.1983

Kolenbrander, P. E., Palmer, R. J. Jr., Rickard, A. H., Jakubovics, N. S., Chalmers, N. I., and Diaz, P. I. (2006). Bacterial interactions and successions during plaque development. *Periodontol. 2000* 42, 47–79. doi: 10.1111/j.1600-0757.2006.00187.x

Kong, X., Liu, J., Cetinbas, M., Sadreyev, R., Koh, M., Huang, H., et al. (2019). New and preliminary evidence on altered oral and gut microbiota in individuals with Autism Spectrum Disorder (ASD): implications for ASD diagnosis and subtyping based on microbial biomarkers. *Nutrients* 11:2128. doi: 10.3390/nu11092128

Koonin, E. V., Makarova, K. S., and Aravind, L. (2001). Horizontal gene transfer in prokaryotes: quantification and classification. *Annu. Rev. Microbiol.* 55, 709–742. doi: 10.1146/annurev.micro.55.1.709

Kreth, J., Merritt, J., Shi, W., and Qi, F. (2005). Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. *J. Bacteriol.* 187, 7193–7203. doi: 10.1128/JB.187.21.7193-7203.2005

Kritchevsky, B., and Seguin, P. (1918). The pathogenesis and treatment of pyorrhea alveolaris. *Dental Cosmos Monthly Rec. Dental Sci.* 60, 781–784.

Krutzik, S. R., Ochoa, M. T., Sieling, P. A., Uematsu, S., Ng, Y. W., Legaspi, A., et al. (2003). Activation and regulation of Toll-like receptors 2 and 1 in human leprosy. *Nat. Med.* 9, 525–532. doi: 10.1038/nm864

Kumar, H., Kawai, T., and Akira, S. (2011). Pathogen recognition by the innate immune system. *Int. Rev. Immunol.* 30, 16–34. doi: 10.3109/08830185.2010.529976

Kumar, P., and Gupta, K. C. (2003). A rapid method for the construction of oligonucleotide arrays. *Bioconjug. Chem.* 14, 507–512. doi: 10.1021/bc025646q

Kumar, S., Ingle, H., Prasad, D. V., and Kumar, H. (2013). Recognition of bacterial infection by innate immune sensors. *Crit. Rev. Microbiol.* 39, 229–246. doi: 10.3109/1040841X.2012.706249

Labonte, A. C., Sung, S. J., Jennelle, L. T., Dandekar, A. P., and Hahn, Y. S. (2017). Expression of scavenger receptor-AI promotes alternative activation of murine macrophages to limit hepatic inflammation and fibrosis. *Hepatology* 65, 32–43. doi: 10.1002/hep.28873

Lai, M. A., Quarles, E. K., López-Yglesias, A. H., Zhao, X., Hajjar, A. M., and Smith, K. D. (2013). Innate immune detection of flagellin positively and negatively regulates salmonella infection. *PLoS ONE* 8:e72047. doi: 10.1371/journal.pone.0072047

Lamell, C. W., Griffen, A. L., Mcclellan, D. L., and Leys, E. J. (2000). Acquisition and colonization stability of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in children. *J. Clin. Microbiol.* 38, 1196–1169. doi: 10.1128/JCM.38.3.1196-1199.2000

Lamont, R. J., and Hajishengallis, G. (2015). Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol. Med.* 21, 172–183. doi: 10.1016/j.molmed.2014.11.004

Lamont, R. J., Koo, H., and Hajishengallis, G. (2018). The oral microbiota: dynamic communities and host interactions. *Nat. Rev. Microbiol.* 16, 745–759. doi: 10.1038/s41579-018-0089-x

Lane, N. (2015). The unseen world: reflections on Leeuwenhoek (1677) 'concerning little animals'. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370, 1–10. doi: 10.1098/rstb.2014.0344

Lang, N. P., Kiel, R. A., and Anderhalden, K. (1983). Clinical and microbiological effects of subgingival restorations with overhanging or clinically perfect margins. *J. Clin. Periodontol.* 10, 563–578. doi: 10.1111/j.1600-051X.1983.tb01295.x

Lauvau, G., and Glaichenhaus, N. (2004). Mini-review: presentation of pathogen-derived antigens *in vivo*. *Eur. J. Immunol.* 34, 913–920. doi: 10.1002/eji.200424944

Li, J., Helmerhorst, E. J., Leone, C. W., Troxler, R. F., Yaskell, T., Haffajee, A. D., et al. (2004). Identification of early microbial colonizers in human dental biofilm. *J. Appl. Microbiol.* 97, 1311–1318. doi: 10.1111/j.1365-2672.2004.02420.x

Li, Y., Messina, C., Bendaoud, M., Fine, D. H., Schreiner, H., and Tsiagbe, V. K. (2010). Adaptive immune response in osteoclastic bone resorption induced by orally administered *Aggregatibacter actinomycetemcomitans* in a rat model of periodontal disease. *Mol. Oral Microbiol.* 25, 275–292. doi: 10.1111/j.2041-1014.2010.00576.x

Liang, S., Domon, H., Hosur, K. B., Wang, M., and Hajishengallis, G. (2009). Age-related alterations in innate immune receptor expression and ability of macrophages to respond to pathogen challenge *in vitro*. *Mech. Ageing Dev.* 130, 538–546. doi: 10.1016/j.mad.2009.06.006

Liu, J., Sun, L., Liu, W., Guo, L., Liu, Z., Wei, X., et al. (2017). A nuclease from *Streptococcus mutans* facilitates biofilm dispersal and escape from killing by neutrophil extracellular traps. *Front. Cell. Infect. Microbiol.* 7:97. doi: 10.3389/fcimb.2017.00097

Lloyd-Price, J., Abu-Ali, G., and Huttenhower, C. (2016). The healthy human microbiome. *Genome Med.* 8:51. doi: 10.1186/s13073-016-0307-y

Locati, M., Mantovani, A., and Sica, A. (2013). Macrophage activation and polarization as an adaptive component of innate immunity. *Adv. Immunol.* 120, 163–184. doi: 10.1016/B978-0-12-417028-5.00006-5

Loe, H., Theilade, E., Jensen, S. B., and Schiott, C. R. (1967). Experimental gingivitis in man. 3. Influence of antibiotics on gingival plaque development. *J. Periodontal. Res.* 2, 282–289. doi: 10.1111/j.1600-0765.1967.tb01901.x

Loesche, W. J. (1976). Chemotherapy of dental plaque infections. *Oral Sci. Rev.* 9, 65–107.

Loesche, W. J. (1993). Bacterial mediators in periodontal disease. *Clin Infect Dis.* 16(Suppl. 4), S203–S210. doi: 10.1093/clinids/16.Supplement_4.S203

Loo, V. G., Bourgault, A. M., Poirier, L., Lamothe, F., Michaud, S., Turgeon, N., et al. (2011). Host and pathogen factors for *Clostridium difficile* infection and colonization. *N. Engl. J. Med.* 365, 1693–1703. doi: 10.1056/NEJMoa1012413

Lu, J., and Claud, E. C. (2019). Connection between gut microbiome and brain development in preterm infants. *Dev. Psychobiol.* 61, 739–751. doi: 10.1002/dev.21806

Lu, J., Lu, L., Yu, Y., Cluette-Brown, J., Martin, C. R., and Claud, E. C. (2018). Effects of intestinal microbiota on brain development in humanized gnotobiotic mice. *Sci. Rep.* 8:5443. doi: 10.1038/s41598-018-23692-w

Macdonald, J. B., Sutton, R. M., Knoll, M. L., Madlener, E. M., and Grainger, R. M. (1956). The pathogenic components of an experimental fusospirochetal infection. *J. Infect. Dis.* 98, 15–20. doi: 10.1093/infdis/98.1.15

Machado, J. C., Figueiredo, C., Canedo, P., Pharoah, P., Carvalho, R., Nabais, S., et al. (2003). A proinflammatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. *Gastroenterology* 125, 364–371. doi: 10.1016/S0016-5085(03)00899-0

Macpherson, A. J., and Harris, N. L. (2004). Interactions between commensal intestinal bacteria and the immune system. *Nat. Rev. Immunol.* 4, 478–485. doi: 10.1038/nri1373

Macpherson, A. J., Yilmaz, B., Limenitakis, J. P., and Ganal-Vonarburg, S. C. (2018). IgA function in relation to the intestinal microbiota. *Annu. Rev. Immunol.* 36, 359–381. doi: 10.1146/annurev-immunol-042617-053238

Mancuso, S., Carlisi, M., Santoro, M., Napolitano, M., Raso, S., and Siragusa, S. (2018). Immunosenescence and lymphomagenesis. *Immun. Ageing* 15:22. doi: 10.1186/s12979-018-0130-y

Mantovani, A., Biswas, S. K., Galdiero, M. R., Sica, A., and Locati, M. (2013). Macrophage plasticity and polarization in tissue repair and remodelling. *J. Pathol.* 229, 176–185. doi: 10.1002/path.4133

Marcinkiewicz, J., Strus, M., and Pasich, E. (2013). Antibiotic resistance: a "dark side" of biofilmassociated chronic infections. *Pol. Arch. Med.* 123, 309–313. doi: 10.20452/pamw.1780

Mari, D., Mannucci, P. M., Coppola, R., Bottasso, B., Bauer, K. A., and Rosenberg, R. D. (1995). Hypercoagulability in centenarians: the paradox of successful aging. *Blood* 85, 3144–3149. doi: 10.1182/blood.V85.11.3144.bloodjournal85113144

Marsh, P. D. (1994). Microbial ecology of dental plaque and its significance in health and disease. *Adv. Dent. Res.* 8, 263–271. doi: 10.1177/08959374940080022001

Marsh, P. D., and Zaura, E. (2017). Dental biofilm: ecological interactions in health and disease. *J Clin Periodontol.* 44(Suppl. 18), S12–S22. doi: 10.1111/jcpe.12679

Martinez, N., Ketheesan, N., West, K., Vallerskog, T., and Kornfeld, H. (2016). Impaired recognition of *Mycobacterium tuberculosis* by alveolar macrophages from diabetic mice. *J. Infect. Dis.* 214, 1629–1637. doi: 10.1093/infdis/jiw436

Mazmanian, S. K., Round, J. L., and Kasper, D. L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 453, 620–625. doi: 10.1038/nature07008

McElhaney, J. E., Zhou, X., Talbot, H. K., Soethout, E., Bleackley, R. C., Granville, D. J., et al. (2012). The unmet need in the elderly: how immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines. *Vaccine* 30, 2060–2067. doi: 10.1016/j.vaccine.2012.01.015

McGhee, J. R., Kiyono, H., Michalek, S. M., and Mestecky, J. (1987). Enteric immunization reveals a T cell network for IgA responses and suggests that humans possess a common mucosal immune system. *Antonie Van Leeuwenhoek* 53, 537–543. doi: 10.1007/BF00415514

Mestecky, J., Kulhavy, R., and Kraus, F. W. (1972). Studies on human secretory immunoglobulin A. II. Subunit structure. *J. Immunol.* 108, 738–747.

Mestecky, J., Mcghee, J. R., Michalek, S. M., Arnold, R. R., Crago, S. S., and Babb, J. L. (1978). Concept of the local and common mucosal immune response. *Adv. Exp. Med. Biol.* 107, 185–192. doi: 10.1007/978-1-4684-3369-2_22

Mestecky, J., Nguyen, H., Czerkinsky, C., and Kiyono, H. (2008). Oral immunization: an update. *Curr. Opin. Gastroenterol.* 24, 713–719. doi: 10.1097/MOG.0b013e32830d58be

Miller, J. F., and Mitchell, G. F. (1968). Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J. Exp. Med.* 128, 801–820. doi: 10.1084/jem.128.4.801

Miller, W. D. (1890). *The Micro-Organisms of the Human Mouth: The Local and General Diseases which are Caused by Them*. Birmingham, AL: Classics of Dentistry Library.

Miossec, P. (2009). IL-17 and Th17 cells in human inflammatory diseases. *Microbes Infect.* 11, 625–630. doi: 10.1016/j.micinf.2009.04.003

Mira, A., Simon-Soro, A., and Curtis, M. A. (2017). Role of microbial communities in the pathogenesis of periodontal diseases and caries. *J. Clin. Periodontol.* 44(Suppl. 18), S23–S38. doi: 10.1111/jcpe.12671

Mitchell, G. F., and Miller, J. F. (1968a). Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given

bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* 128, 821–837. doi: 10.1084/jem.128.4.821

Mitchell, G. F., and Miller, J. F. (1968b). Immunological activity of thymus and thoracic-duct lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 59, 296–303. doi: 10.1073/pnas.59.1.296

Moldoveanu, Z., Russell, M. W., Wu, H. Y., Huang, W. Q., Compans, R. W., and Mestecky, J. (1995). Compartmentalization within the common mucosal immune system. *Adv. Exp. Med. Biol.* 371A, 97–101. doi: 10.1007/978-1-4615-1941-6_17

Moore, W. E. C., and Moore, L. V. H. (1994). The bacteria of periodontal disease. *Periodontol. 2000* 5, 66–77. doi: 10.1111/j.1600-0757.1994.tb00019.x

Moutsopoulos, N. M., Chalmers, N. I., Barb, J. J., Abusleme, L., Greenwell-Wild, T., Dutzan, N., et al. (2015). Subgingival microbial communities in Leukocyte Adhesion Deficiency and their relationship with local immunopathology. *PLoS Pathog.* 11:e1004698. doi: 10.1371/journal.ppat.1004698

Moutsopoulos, N. M., and Konkel, J. E. (2018). Tissue-specific immunity at the oral mucosal barrier. *Trends Immunol.* 39, 276–287. doi: 10.1016/j.it.2017.08.005

Mueller, N. T., Bakacs, E., Combellick, J., Grigoryan, Z., and Dominguez-Bello, M. G. (2015). The infant microbiome development: mom matters. *Trends Mol. Med.* 21, 109–117. doi: 10.1016/j.molmed.2014.12.002

Mukherjee, S., Sinha, D., Ghosh, A. K., and Biswas, T. (2014). Bacterial ligand stimulates TLR2-dependent chemokines of colon cell. *Immunobiology* 219, 350–356. doi: 10.1016/j.imbio.2013.12.002

Negi, S., Das, D. K., Pahari, S., Nadeem, S., and Agrewala, J. N. (2019). Potential role of gut microbiota in induction and regulation of innate immune memory. *Front. Immunol.* 10:2441. doi: 10.3389/fimmu.2019.02441

Nibali, L., Di Iorio, A., Onabolu, O., and Lin, G. H. (2016). Periodontal infectogenomics: systematic review of associations between host genetic variants and subgingival microbial detection. *J. Clin. Periodontol.* 43, 889–900. doi: 10.1111/jcpe.12600

Nibali, L., Henderson, B., Sadiq, S. T., and Donos, N. (2014). Genetic dysbiosis: the role of microbial insults in chronic inflammatory diseases. *J. Oral Microbiol.* 6, 1–10. doi: 10.3402/jom.v6.22962

Nishiguchi, M., Matsumoto, M., Takao, T., Hoshino, M., Shimonishi, Y., Tsuji, S., et al. (2001). *Mycoplasma fermentans* lipoprotein M161Ag-induced cell activation is mediated by Toll-like receptor 2: role of N-terminal hydrophobic portion in its multiple functions. *J. Immunol.* 166, 2610–2616. doi: 10.4049/jimmunol.166.4.2610

Nutsch, K. M., and Hsieh, C. S. (2012). T cell tolerance and immunity to commensal bacteria. *Curr. Opin. Immunol.* 24, 385–391. doi: 10.1016/j.coi.2012.04.009

Offenbacher, S., Divaris, K., Barros, S. P., Moss, K. L., Marchesan, J. T., Morelli, T., et al. (2016). Genome-wide association study of biologically informed periodontal complex traits offers novel insights into the genetic basis of periodontal disease. *Hum. Mol. Genet.* 25, 2113–2129. doi: 10.1093/hmg/ddw069

Okamoto, K., and Takayanagi, H. (2019). Osteoimmunology. *Cold Spring Harb. Perspect. Med.* 9, 1–28. doi: 10.1101/cshperspect.a031245

Olivier, M., Asmis, R., Hawkins, G. A., Howard, T. D., and Cox, L. A. (2019). The need for multi-omics biomarker signatures in precision medicine. *Int. J. Mol. Sci.* 20:4781. doi: 10.3390/ijms20194781

O'Malley, M. A. (2007). The nineteenth century roots of 'everything is everywhere'. *Nat. Rev. Microbiol.* 5, 647–651. doi: 10.1038/nrmicro1711

Page, R. C., and Schroeder, H. E. (1976). Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab. Invest.* 34, 235–249.

Pandey, A., Cleary, D. W., Laver, J. R., Gorringe, A., Deasy, A. M., Dale, A. P., et al. (2018). Microevolution of *Neisseria lactamica* during nasopharyngeal colonisation induced by controlled human infection. *Nat. Commun.* 9:4753. doi: 10.1038/s41467-018-07235-5

Papapanou, P. N., Sanz, M., Buduneli, N., Dietrich, T., Feres, M., Fine, D. H., et al. (2018). Periodontitis: consensus report of workgroup 2 of the 2017 world workshop on the classification of periodontal and peri-implant diseases and conditions. *J. Periodontol.* 89(Suppl. 1), S173–S182. doi: 10.1002/JPER.17-0721

Park, O. J., Yi, H., Jeon, J. H., Kang, S. S., Koo, K. T., Kum, K. Y., et al. (2015). Pyrosequencing analysis of subgingival microbiota in distinct periodontal conditions. *J. Dent. Res.* 94, 921–927. doi: 10.1177/0022034515583531

Peter, M. E., Kubarenko, A. V., Weber, A. N., and Dalpke, A. H. (2009). Identification of an N-terminal recognition site in TLR9 that contributes to CpG-DNA-mediated receptor activation. *J. Immunol.* 182, 7690–7697. doi: 10.4049/jimmunol.0900819

Petersen, C., and Round, J. L. (2014). Defining dysbiosis and its influence on host immunity and disease. *Cell. Microbiol.* 16, 1024–1033. doi: 10.1111/cmi.12308

Pinna, R., Campus, G., Cumbo, E., Mura, I., and Milia, E. (2015). Xerostomia induced by radiotherapy: an overview of the physiopathology, clinical evidence, and management of the oral damage. *Ther. Clin. Risk Manag.* 11, 171–188. doi: 10.2147/TCRM.S70652

Pirofski, L. A., and Casadevall, A. (2008). The damage-response framework of microbial pathogenesis and infectious diseases. *Adv. Exp. Med. Biol.* 635, 135–146. doi: 10.1007/978-0-387-09550-9_11

Polechová, J., and Storch, D. (2019). "Ecological niche," in *Encyclopedia of Ecology, 2nd Edn,* ed B. Fath (Oxford: Elsevier), 72–80. doi: 10.1016/B978-0-12-409548-9.11113-3

Preiano, M., Maggisano, G., Lombardo, N., Montalcini, T., Paduano, S., Pelaia, G., et al. (2016). Influence of storage conditions on MALDI-TOF MS profiling of gingival crevicular fluid: Implications on the role of S100A8 and S100A9 for clinical and proteomic based diagnostic investigations. *Proteomics* 16, 1033–1045. doi: 10.1002/pmic.201500328

Qian, F., Wang, X., Zhang, L., Lin, A., Zhao, H., Fikrig, E., et al. (2011). Impaired interferon signaling in dendritic cells from older donors infected *in vitro* with West Nile virus. *J. Infect. Dis.* 203, 1415–1424. doi: 10.1093/infdis/jir048

Raju, T. N. (1999). The Nobel chronicles. 1972: gerald M Edelman (b 1929) and Rodney R Porter (1917-85). *Lancet* 354:1040. doi: 10.1016/S0140-6736(05)76658-7

Ram-Mohan, N., and Meyer, M. M. (2020). Comparative metatranscriptomics of periodontitis supports a common polymicrobial shift in metabolic function and identifies novel putative disease-associated ncRNAs. *Front. Microbiol.* 11:482. doi: 10.3389/fmicb.2020.00482

Relman, D. A. (2012). The human microbiome: ecosystem resilience and health. *Nutr Rev.* 70(Suppl. 1), S2–S9. doi: 10.1111/j.1753-4887.2012.00489.x

Ribeiro, C. M., Hermsen, T., Taverne-Thiele, A. J., Savelkoul, H. F., and Wiegertjes, G. F. (2010). Evolution of recognition of ligands from Gram-positive bacteria: similarities and differences in the TLR2-mediated response between mammalian vertebrates and teleost fish. *J. Immunol.* 184, 2355–2368. doi: 10.4049/jimmunol.0900990

Riedel, S. (2005). Edward Jenner and the history of smallpox and vaccination. *Proc. Bayl. Univ. Med. Cent.* 18, 21–25. doi: 10.1080/08998280.2005.11928028

Rook, G. A. (2010). 99th Dahlem conference on infection, inflammation and chronic inflammatory disorders: darwinian medicine and the 'hygiene' or 'old friends' hypothesis. *Clin. Exp. Immunol.* 160, 70–79. doi: 10.1111/j.1365-2249.2010.04133.x

Rook, G. A., Raison, C. L., and Lowry, C. A. (2014). Microbial 'old friends', immunoregulation and socioeconomic status. *Clin. Exp. Immunol.* 177, 1–12. doi: 10.1111/cei.12269

Rook, G. A. W., Lowry, C. A., and Raison, C.L. (2013). Microbial "Old Friends" immunoregulation and stress resilience. *Evol. Med. Public Health* 2013, 46–64. doi: 10.1093/emph/eot004

Rosebury, T., Foley, G., and Greenberg, S. (1934). Studies of lactobacilli in relation to caries in rats. 2. Attempt to immunize rats, on cariesproducing diets, against lactobacilli. *J. Dent. Res.* 14, 231–232.

Rosebury, T., Linton, R. W., and Buchbinder, L. (1929). A comparative study of dental aciduric organisms and *Lactobacillus Acidophilus*. *J. Bacteriol.* 18, 395–412. doi: 10.1128/JB.18.6.395-412.1929

Rosenow, E. C. (1919). Studies on elective localization focal infection with special reference to oral sepsis'. *J. Dent. Res.* 1, 205–267. doi: 10.1177/00220345190010030101

Rosenow, E. C. (1930). Elective localization of streptococci. *Br. Med. J.* 1, 1100–1101. doi: 10.1136/bmj.1.3623.1100

Rosenzweig, H. L., Galster, K., Vance, E. E., Ensign-Lewis, J., Nunez, G., Davey, M. P., et al. (2011). NOD2 deficiency results in increased susceptibility to peptidoglycan-induced uveitis in mice. *Invest. Ophthalmol. Vis. Sci.* 52, 4106–4112. doi: 10.1167/iovs.10-6263

Round, J. L., and Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9, 313–323. doi: 10.1038/nri2515

Schett, G. (2016). [Osteoimmunology]. *Z. Rheumatol.* 75, 531–533. doi: 10.1007/s00393-016-0144-9

Shafquat, A., Joice, R., Simmons, S. L., and Huttenhower, C. (2014). Functional and phylogenetic assembly of microbial communities in the human microbiome. *Trends Microbiol.* 22, 261–266. doi: 10.1016/j.tim.2014.01.011

Shanley, D. P., Aw, D., Manley, N. R., and Palmer, D. B. (2009). An evolutionary perspective on the mechanisms of immunosenescence. *Trends Immunol.* 30, 374–381. doi: 10.1016/j.it.2009.05.001

Sharma, H., Turner, C. E., Siggins, M. K., El-Bahrawy, M., Pichon, B., Kearns, A., et al. (2019). Toxic shock syndrome toxin 1 evaluation and antibiotic impact in a transgenic model of staphylococcal soft tissue infection. *mSphere* 4:e00665-19. doi: 10.1128/mSphere.00665-19

Shin, M. S., Kim, Y. G., Shin, Y. J., Ko, B. J., Kim, S., and Kim, H. D. (2019). Deep sequencing salivary proteins for periodontitis using proteomics. *Clin. Oral Investig.* 23, 3571–3580. doi: 10.1007/s00784-018-2779-1

Silva, L. M., Brenchley, L., and Moutsopoulos, N. M. (2019). Primary immunodeficiencies reveal the essential role of tissue neutrophils in periodontitis. *Immunol. Rev.* 287, 226–235. doi: 10.1111/imr.12724

Silva, N., Abusleme, L., Bravo, D., Dutzan, N., Garcia-Sesnich, J., Vernal, R., et al. (2015). Host response mechanisms in periodontal diseases. *J. Appl. Oral Sci.* 23, 329–355. doi: 10.1590/1678-775720140259

Singh, M. P., Saxena, M., Saimbi, C. S., Siddiqui, M. H., and Roy, R. (2019). Post-periodontal surgery propounds early repair salivary biomarkers by (1)H NMR based metabolomics. *Metabolomics* 15:141. doi: 10.1007/s11306-019-1593-3

Singleton, T. E., Massari, P., and Wetzler, L. M. (2005). Neisserial porin-induced dendritic cell activation is MyD88 and TLR2 dependent. *J. Immunol.* 174, 3545–3550. doi: 10.4049/jimmunol.174.6.3545

Smith, C. J., Sayles, H., Mikuls, T. R., and Michaud, K. (2011). Minocycline and doxycycline therapy in community patients with rheumatoid arthritis: prescribing patterns, patient-level determinants of use, and patient-reported side effects. *Arthritis Res. Ther.* 13:R168. doi: 10.1186/ar3491

Socransky, S. S. (1979). Criteria for the infectious agents in dental caries and periodontal disease. *J. Clin. Periodontol.* 6, 16–21. doi: 10.1111/j.1600-051X.1979.tb02114.x

Socransky, S. S., and Haffajee, A. D. (1991). Microbial mechanisms in the pathogenesis of destructive periodontal diseases: a critical assessment. *J. Periodont. Res.* 26, 195–212. doi: 10.1111/j.1600-0765.1991.tb01646.x

Socransky, S. S., and Haffajee, A. D. (1994). Evidence of bacterial etiology: a historical perspective. *Periodontol. 2000* 5, 7–25. doi: 10.1111/j.1600-0757.1994.tb00016.x

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., and Kent, R. L. Jr. (1998). Microbial complexes in subgingival plaque. *J. Clin. Periodontol.* 25, 134–144. doi: 10.1111/j.1600-051X.1998.tb02419.x

Socransky, S. S., Haffajee, A. D., Smith, G. L., and Dzink, J. L. (1987). Difficulties encountered in the search for the etiologic agents of destructive periodontal diseases. *J. Clin. Periodontol.* 14, 588–593. doi: 10.1111/j.1600-051X.1987.tb01520.x

Socransky, S. S., and Manganiello, S. D. (1971). The oral microbiota of man from birth to senility. *J. Periodontol.* 42, 485–496. doi: 10.1902/jop.1971.42.8.485

Socransky, S. S., Smith, C., Martin, L., Paster, B. J., Dewhirst, F. E., and Levin, A. E. (1994). "Checkerboard" DNA-DNA hybridization. *BioTechniques* 17, 788–792.

Sokol, C. L., and Luster, A. D. (2015). The chemokine system in innate immunity. *Cold Spring Harb Perspect Biol* 7.

Sommer, F., and Backhed, F. (2013). The gut microbiota–masters of host development and physiology. *Nat. Rev. Microbiol.* 11, 227–238. doi: 10.1038/nrmicro2974

Sprent, J. (2017). T cell–B cell collaboration. *Nat. Rev. Immunol.* 17, 532–532. doi: 10.1038/nri.2017.62

Sridharan, A., Esposo, M., Kaushal, K., Tay, J., Osann, K., Agrawal, S., et al. (2011). Age-associated impaired plasmacytoid dendritic cell functions lead to decreased CD4 and CD8 T cell immunity. *Age* 33, 363–376. doi: 10.1007/s11357-010-9191-3

Stebbins, C. E., and Galán, J. E. (2001). Structural mimicry in bacterial virulence. *Nature* 412, 701–705. doi: 10.1038/35089000

Stephan, R. M., and Miller, B. F. (1943). A quantitative method for evaluating physical and chemical agents which modify production of acids in bacterial plaques on human teeth. *J. Dent. Res.* 22, 45–51. doi: 10.1177/00220345430220010601

Strohmeier, G. R., and Fenton, M. J. (1999). Roles of lipoarabinomannan in the pathogenesis of tuberculosis. *Microbes Infect.* 1, 709–717. doi: 10.1016/S1286-4579(99)80072-0

Sullivan, A., Edlund, C., and Nord, C. E. (2001). Effect of antimicrobial agents on the ecological balance of human microflora. *Lancet Infect. Dis.* 1, 101–114. doi: 10.1016/S1473-3099(01)00066-4

Sun, L., Zhang, X., Zhang, Y., Zheng, K., Xiang, Q., Chen, N., et al. (2019). Antibiotic-induced disruption of gut microbiota alters local metabolomes and immune responses. *Front. Cell. Infect. Microbiol.* 9:99. doi: 10.3389/fcimb.2019.00099

Syndergaard, B., Al-Sabbagh, M., Kryscio, R. J., Xi, J., Ding, X., Ebersole, J. L., et al. (2014). Salivary biomarkers associated with gingivitis and response to therapy. *J. Periodontol.* 85, e295–e303. doi: 10.1902/jop.2014.130696

Takahashi, N., and Nyvad, B. (2011). The role of bacteria in the caries process: ecological perspectives. *J. Dent. Res.* 90, 294–303. doi: 10.1177/0022034510379602

Takahashi, N., and Nyvad, B. (2016). Ecological hypothesis of dentin and root caries. *Caries Res.* 50, 422–431. doi: 10.1159/000447309

Takeuchi, O., Hoshino, K., and Akira, S. (2000). Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J. Immunol.* 165, 5392–5396. doi: 10.4049/jimmunol.165.10.5392

Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., et al. (2002). Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J. Immunol.* 169, 10–14. doi: 10.4049/jimmunol.169.1.10

Teng, T. S., Ji, A. L., Ji, X. Y., and Li, Y. Z. (2017). Neutrophils and immunity: from bactericidal action to being conquered. *J. Immunol. Res.* 2017:9671604. doi: 10.1155/2017/9671604

Terashima, A., and Takayanagi, H. (2018). Overview of osteoimmunology. *Calcif. Tissue Int.* 102, 503–511. doi: 10.1007/s00223-018-0417-1

Tordesillas, L., and Berin, M. C. (2018). Mechanisms of oral tolerance. *Clin. Rev. Allergy Immunol.* 55, 107–117. doi: 10.1007/s12016-018-8680-5

Trautmann, S., Barghash, A., Fecher-Trost, C., Schalkowsky, P., Hannig, C., Kirsch, J., et al. (2019). Proteomic analysis of the initial oral pellicle in caries-active and caries-free individuals. *Proteomics Clin. Appl.* 13:e1800143. doi: 10.1002/prca.201800143

Treerat, P., Redanz, U., Redanz, S., Giacaman, R. A., Merritt, J., and Kreth, J. (2020). Synergism between *Corynebacterium* and *Streptococcus sanguinis* reveals new interactions between oral commensals. *ISME J.* 14, 1154–1169. doi: 10.1038/s41396-020-0598-2

Tsiagbe, V. K., and Fine, D. H. (2012). The impact of bacteria-induced adaptive immune responses in periodontal disease. *Periodontal Dis. A Clin. Guide* 93, 93–106. doi: 10.5772/26831

Uzel, N. G., Teles, F. R., Teles, R. P., Song, X. Q., Torresyap, G., Socransky, S. S., et al. (2011). Microbial shifts during dental biofilm re-development in the absence of oral hygiene in periodontal health and disease. *J. Clin. Periodontol.* 38, 612–620. doi: 10.1111/j.1600-051X.2011.01730.x

Uzozie, A. C., and Aebersold, R. (2018). Advancing translational research and precision medicine with targeted proteomics. *J. Proteomics* 189, 1–10. doi: 10.1016/j.jprot.2018.02.021

van Crevel, R., Ottenhoff, T. H., and Van Der Meer, J. W. (2002). Innate immunity to *Mycobacterium tuberculosis*. *Clin. Microbiol. Rev.* 15, 294–309. doi: 10.1128/CMR.15.2.294-309.2002

van der Hoeven, J. S., and Franken, H. C. (1984). Effect of fluoride on growth and acid production by *Streptococcus mutans* in dental plaque. *Infect. Immun.* 45, 356–359. doi: 10.1128/IAI.45.2.356-359.1984

Van Dyke, T. E. (2017). Pro-resolving mediators in the regulation of periodontal disease. *Mol. Aspects Med.* 58, 21–36. doi: 10.1016/j.mam.2017.04.006

Vila, T., Rizk, A. M., Sultan, A. S., and Jabra-Rizk, M. A. (2019). The power of saliva: antimicrobial and beyond. *PLoS Pathog.* 15:e1008058. doi: 10.1371/journal.ppat.1008058

Waerhaug, J. (1956). Effect of rough surfaces upon gingival tissue. *J. Dent. Res.* 35, 323–325. doi: 10.1177/00220345560350022601

Wang, K., Wang, Y., Wang, X., Ren, Q., Han, S., Ding, L., et al. (2018). Comparative salivary proteomics analysis of children with and without dental caries using the iTRAQ/MRM approach. *J. Transl. Med.* 16:11. doi: 10.1186/s12967-018-1388-8

Weng, N. P. (2006). Aging of the immune system: how much can the adaptive immune system adapt? *Immunity* 24, 495–499. doi: 10.1016/j.immuni.2006.05.001

Williams, R. C., Barnett, A. H., Claffey, N., Davis, M., Gadsby, R., Kellett, M., et al. (2008). The potential impact of periodontal disease on general health: a consensus view. *Curr. Med. Res. Opin.* 24, 1635–1643. doi: 10.1185/03007990802131215

Willing, B. P., Russell, S. L., and Finlay, B. B. (2011). Shifting the balance: antibiotic effects on host-microbiota mutualism. *Nat. Rev. Microbiol.* 9, 233–243. doi: 10.1038/nrmicro2536

Willmann, M., Vehreschild, M., Biehl, L. M., Vogel, W., Dorfel, D., Hamprecht, A., et al. (2019). Distinct impact of antibiotics on the gut microbiome and resistome: a longitudinal multicenter cohort study. *BMC Biol.* 17:76. doi: 10.1186/s12915-019-0692-y

Xu, X., He, J., Xue, J., Wang, Y., Li, K., Zhang, K., et al. (2015). Oral cavity contains distinct niches with dynamic microbial communities. *Environ. Microbiol.* 17, 699–710. doi: 10.1111/1462-2920.12502

Yost, S., Duran-Pinedo, A. E., Teles, R., Krishnan, K., and Frias-Lopez, J. (2015). Functional signatures of oral dysbiosis during periodontitis progression revealed by microbial metatranscriptome analysis. *Genome Med.* 7:27. doi: 10.1186/s13073-015-0231-6

Yu, X., Feng, B., He, P., and Shan, L. (2017). From chaos to harmony: responses and signaling upon microbial pattern recognition. *Annu. Rev. Phytopathol.* 55, 109–137. doi: 10.1146/annurev-phyto-080516-035649

Zhang, F., Tanaka, H., Kawato, T., Kitami, S., Nakai, K., Motohashi, M., et al. (2011). Interleukin-17A induces cathepsin K and MMP-9 expression in osteoclasts via celecoxib-blocked prostaglandin E2 in osteoblasts. *Biochimie* 93, 296–305. doi: 10.1016/j.biochi.2010.10.001

Zhang, S., Yu, N., and Arce, R. M. (2020). Periodontal inflammation: integrating genes and dysbiosis. *Periodontol. 2000* 82, 129–142. doi: 10.1111/prd.12267

Zhou, J., Yao, Y., Jiao, K., Zhang, J., Zheng, X., Wu, F., et al. (2017). Relationship between gingival crevicular fluid microbiota and cytokine profile in periodontal host homeostasis. *Front. Microbiol.* 8:2144. doi: 10.3389/fmicb.2017.02144

Zhou, L. N., Bi, C. S., Gao, L. N., An, Y., Chen, F., and Chen, F. M. (2019). Macrophage polarization in human gingival tissue in response to periodontal disease. *Oral Dis.* 25, 265–273. doi: 10.1111/odi.12983

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Bacteroides thetaiotaomicron Ameliorates Experimental Allergic Airway Inflammation *via* Activation of ICOS$^+$Tregs and Inhibition of Th2 Response

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Inhibition of allergic airway diseases (AAD) by immunomodulation of the adaptive immune system through restoration of the enteric dysbiosis is an emerging therapeutic strategy. Patients with allergic rhinitis (n = 6) and healthy controls (n = 6) were enrolled, and gut microbiome composition analysis was performed by 16S rDNA sequencing. We also established an ovalbumin (OVA)-induced allergic airway inflammation murine model. Dysbiosis of the gut flora was observed in both AAD patients and the mice, with the decrease of the biodiversity and the quantity of the Bacteroidetes phylum. Oral application of *Bacteroides (B.) thetaiotaomicron* ameliorated the symptoms of OVA-induced airway hyperresponsiveness (AHR) and attenuated the airway inflammation in mice. In addition, nasal lavage fluid (NALF) and bronchoalveolar lavage fluid (BALF) from AAD mice orally administered with *B. thetaiotaomicron* showed reduced numbers of immune cells, and diminished secretion of T helper (Th)-2 cytokines (IL-4, IL-5, and IL-13) compared with the corresponding control mice, whereas the levels of Th1 cytokineIFN-γ was not changed in both the groups. When *B. thetaiotaomicron* was co-administered with metronidazole in AAD mice, the immunomodulatory effect was weakened and the allergic inflammatory response was aggravated. The ratios of CD4$^+$Foxp3$^+$ cells, CD4$^+$ICOS$^+$ T cells, CD4$^+$ICOS$^+$ Foxp3$^+$ regulatory T cells, and IL-10-expressing CD4$^+$Foxp3$^+$ cells were increased in lymphocytes of spleen, mesenteric, and cervical lymph nodes of AAD mice administrated with *B. thetaiotaomicron*. Therefore, our data indicate that oral administration of *B. thetaiotaomicron* effectively inhibited the development of AAD in murine model; inhibition was mediated by the activation of Tregs and inhibition of Th2 response without promoting a Th1 response.

Keywords: allergic airway diseases, 16s rRNA gene, dysbiosis, *Bacteroides thetaiotaomicron*, regulatory T cells

INTRODUCTION

Allergic rhinitis and asthma are different manifestations of allergic airway disease (AAD) in upper and lower respiratory tracts, having similar pathogenesis and clinical characteristics (1). As per epidemiological investigations, in recent years the incidence of respiratory allergic inflammatory diseases has significantly increased with the change in living environment in many regions or countries including China. At present, there are at least 300 million asthma patients in the world, and the incidence of allergic rhinitis is as high as 20% (2, 3). However, specific underlying causes of this disease are still unknown.

AAD is immunologically dominated by the T-helper type 2 (Th2) cells in the respiratory tract (1). A re-exposure to specific antigens such as pollen and dust mites, stimulates IgE production by the plasma cells and activates the sensitized mast cells degranulation. This results in the release of inflammatory mediators, such as leukotrienes, histamine, and prostaglandins, inducing T-helper type 1 (Th1)/Th2 immune-imbalance. Th2 cells assemble and release a large amount of IL-4, IL-5, IL-13, and other inflammatory cytokines, causing the infiltration of eosinophils in the respiratory tract mucosa to evoke the clinical symptoms of allergy (1, 4).

Th2 polarization and abnormal levels of IgE mirror the dysfunctional immune regulatory system in AAD patients. The regulatory T cells (Tregs), regulatory B cells (Bregs), tolerogenic dendritic cells (DCs), and immuneregulatory cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-10 comprise the immune regulatory system (5–7). Inducible co-stimulatory molecule (ICOS) (CD278), a type of co-stimulatory molecule, is expressed by activated T cells and Tregs (8). ICOS enhances the proliferation, function, and survival of Tregs and plays an important role in a variety of autoimmune diseases and allergic diseases especially asthma (9, 10).

Adequate microbial stimuli from commensal microbiota are indispensable for the maintenance of immunefunctions in the body. Dysbiosis disturbs the immune system, potentially leading to inflammation. Studies have associated early dysbiosis of the gut microbiome with allergies (11, 12). A pivotal study reported by Noverr et al. demonstrated that allergies can develop as a consequence of an altered gut microbiota, suggesting that alterations in the gut microbiota can facilitate an immunological state that is predisposed to respiratory allergies (13). In addition, Vital et al. found that a locally induced pulmonary allergic response is affecting the composition of the intestinal microbiome, indicating bidirectional gut-lung communications (14). One of the important strategies of AAD inhibition is immunomodulation of the adaptive immune system through restoration of enteric dysbiosis (15). In the present study, we investigated the fecal microbiome of AAD based on 16S rDNA sequencing and found a significantly lower abundance of the Bacteroidetes phylum in both AAD patients and mice. *B. thetaiotaomicron* is one of the most abundant bacteria in gut flora, which belongs to the genus Bacteroides. We were specifically interested in exploring the potential immunomodulatory effect of orally administered *B. thetaiotaomicron* on treating AAD.

MATERIALS AND METHODS

Patients and Sample Collection

The study guidelines were approved by the Affiliated Hospital of Qingdao University Review Board and the Affiliated Eye, Ear, Nose, and Throat Hospital of Fudan University Review Board (IRB No. QYFYWZLL26053). Patients aged from 18 to 65 years were recruited from February 2013 to April 2014 and categorized into allergic rhinitis (n = 6) or healthy control (n = 6) groups. Fresh stools were collected and immediately stored at −80°C until further use. Prior to sampling none of the patients received antibiotics for at least 1 month. Pregnant and lactating women and patients with organic heart disease, asthma, or abnormal liver or kidney functions were excluded.

Animals

Female BALB/c (5 to 6 weeks) were purchased from Beijing Vital River Laboratory Animal Technologies Co. Ltd (Beijing, China) and maintained under specific-pathogen-free (SPF) conditions. The animal experiment procedures were approved by the Animal Care Committee of Qingdao University and Fudan University (IACUC No. AHQU20170831).

OVA Sensitization and Challenge

Mice were intraperitoneally (i.p.) injected with 200 μl PBS or 40 μg OVA (Sigma) in 2 mg/100 μl $Al(OH)_3$ gel (Thermo) suspended in PBS on days 1, 3, 5, 7, 9, 11, and 13 (sensitization). This was followed by topical application of 20 μl PBS or 5% OVA solution once daily *via* nasal drops from day 21 to 27 (challenge). Mouse feces were collected 24h after challenge.

16S rRNA Gene Amplification and Sequencing

Extraction of bacterial DNA from 0.5g of human or mouse feces was performed by a FastDNA Spin Kit (OMEGA) following the manufacturer's instructions. The variable region V3–V4 was amplified by polymerase chain reaction (PCR) using bacteria/archaeal primers 341F/805R with barcodes. The amplified DNA was quantified using a Qubit 2.0 DNA detection kit (Thermo Scientific). 10 ng of DNA was taken from each sample, and the final sequencing concentration was 20 pmol. Microbial metagenomic 16S rDNA sequencing was performed.

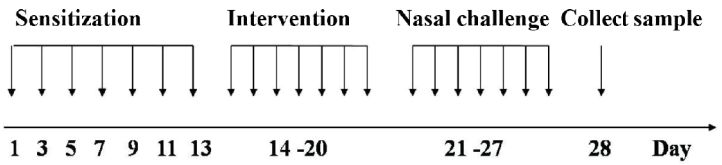
B. thetaiotaomicron Treatment

The *B. thetaiotaomicron* strain 29148 was obtained from ATCC (USA). Prior to oral administration of bacteria in mice, the concentration of the bacteria was adjusted to 10^6 or 10^8 colony forming units (CFUs)/ml with PBS. After OVA sensitizing, PBS, *B. thetaiotaomicron* (10^6 CFU/ml, 10^8 CFU/ml), or *B. thetaiotaomicron* (10^8 CFU/ml) combined with 0.5 mg metronidazole was administered by oral gavage from day 14 to 20 once per day, respectively (**Figure 1**).

Observation of Nasal Symptoms

Sneeze numbers were counted by reviewing the videos of daily activities of the animals in an observation cage. Counting was

A



B

Group	Control	10^8BT	OVA	OVA/10^6BT	OVA/10^8BT	OVA/10^8BT+Met
Number of animals	6	6	6	6	6	6*
Sensitization(i.p.)	PBS	PBS	OVA	OVA	OVA	OVA
Intervention (i.g.)	PBS	10^8CFU	PBS	10^6CFU	10^8CFU	10^8CFU +Met
Nasal challenge (i.n.)	PBS	PBS	OVA	OVA	OVA	OVA

BT, *Bacteroides thetaiotaomicron*; Met, metronidazole; OVA, ovalbumin ; CFU, colony forming units; i.p., intraperitoneal; i.g., intragastrical; i.n., intranasal
*One of the mice died of intragastric insertion of the trachea during intervention.

FIGURE 1 | The experimental protocol **(A)** and different groups **(B)**. Mice were given *B. thetaiotaomicron* daily from Day 14–20, with OVA-sensitization on Day 1, 3, 5, 7, 9, 11, and 13, and nasal challenge with 5% OVA from Day 21–27. Then mice were sacrificed on Day 28 to collect tissues or samples for experimental analysis. Mice were divided into six different groups in this work. Each experimental group consisted of six mice.

immediately performed for a period of 10 min after the final intranasal challenge.

Cell Assays for NALF and BALF

After partial tracheal resection, the nasal and lungs were perfused three times with 0.5 ml PBS, and NALF and BALF samples were collected 24 h after the last OVA challenge. The samples were centrifuged at 3,000 rpm for 5 min, and the lavage supernatant was collected for enzyme-linked immunosorbent assay (ELISA). The cell pellets were re-suspended in 0.1 ml PBS, and the cells were counted using a hemocytometer. Cytospin preparations and Wright-Giemsa staining were then performed for different cell counts followed by counting the absolute numbers of each cell type under a light microscope (Leica).

ELISA

Mouse serum samples were prepared 24 h after the last OVA challenge, and OVA-specific IgE and IgG1 levels were measured using commercially available ELISA kits (Chondrex). Concentrations of cytokines IFN-γ, IL-4, IL-5, IL-10, and IL-13 in both NALF and BALF samples were assessed by ELISA as per the manufacturer's instructions (Merck Millipore).

Histopathological Analysis

The nasal and lung were excised 24 h after the final challenge and fixed in 10% formalin. The fixed nasal was further decalcified with 10% EDTA for 7 days. The samples were embedded in paraffin, and 4-μm sections were cut and fixed on the glass slides. The slides were deparaffinized and stained with HE or toluidine blue. For inflammation scoring of the lungs, a reproducible scoring system was used as previously described (16). Five fields per sample were carefully examined for calculating number of eosinophils or mast cells in the nasal mucosa and lungs.

Fluorescence-Activated Cell Sorting (FACS) Analysis

Mesenteric lymph nodes (LNs), cervical LNs and spleen cells were collected 24 h after the final challenge. These cells were then washed with 1 × PBS and incubated with anti-CD16/CD32 mAb (eBioscience) for 15 min to block nonspecific Ab binding. Cells were subsequently stained with PercpCy5.5–anti-CD25 (eBioscience), FITC–anti-CD4 (eBioscience), and PE-anti-ICOS (eBioscience) at 4°C for 30 min. The cells were fixed and permeabilized for 30 min after washing. APC-anti-Foxp3 mAb (eBioscience) was added to the cells, and the cells incubated for another 30 min at 4°C. Data were recorded by a FACS Calibur flow cytometer (BD Biosciences) and analyzed using FlowJo software. For intracellular cytokine staining of IL-10, cells isolated from mesenteric LNs, cervical LNs and spleens were stimulated for 4h with ionomycin (250 ng/ml) and PMA (10 ng/ml) in RPMI 1640 medium containing 10% FBS, 1% streptomycin and penicillin, 2mM L-glutamine and 10mM HEPEs in 5% CO_2 at 37°C. Then the cells were stained with PercpCy5.5–anti-CD25 (eBioscience), FITC–anti-CD4 (eBioscience), APC-anti-Foxp3 (eBioscience), and PE-anti-IL-10 (eBioscience).

Statistical Analysis

Data were presented as mean ± SD. All data analyses were performed with SPSS20.0 and GraphPad Prism 6.0. For multiple comparisons of data between more than two groups, we used

one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A p-value < 0.05 was considered statistically significant.

RESULTS

Bacteroides Are Reduced in Allergic Rhinitis Patients and Mice

Stool specimens collected from patients with allergic rhinitis and healthy controls. 16S rDNA technology was used to detect the diversity and abundance of intestinal microflora in patients with allergic rhinitis. We found that the Simpson rarefaction in allergic rhinitis patients significantly increased as compared with that in healthy controls, indicating an expected decrease of biodiversity in allergic rhinitis patients (**Figure 2A**). The bacterial composition and abundance at the phylum and genus levels are shown in **Figures 2B** and **C** respectively. In healthy controls, taxonomic classification suggested a high abundance of phylum Firmicutes followed by phylum Bacteroidetes, while an expected lower abundance of Bacteroidetes was found in allergic rhinitis patients at phylum level (**Figure 2B**). At the genus level, abundance of Bacteroides in patients with rhinitis was significantly less as compared with that in healthy controls (**Figure 2C**). We obtained similar results including decreased biodiversity (**Figure 3A**), lower abundance of phylum Bacteroidetes (**Figure 3B**) and Bacteroides (**Figure 3C**) in the gut microflora of a murine model of OVA-induced allergic

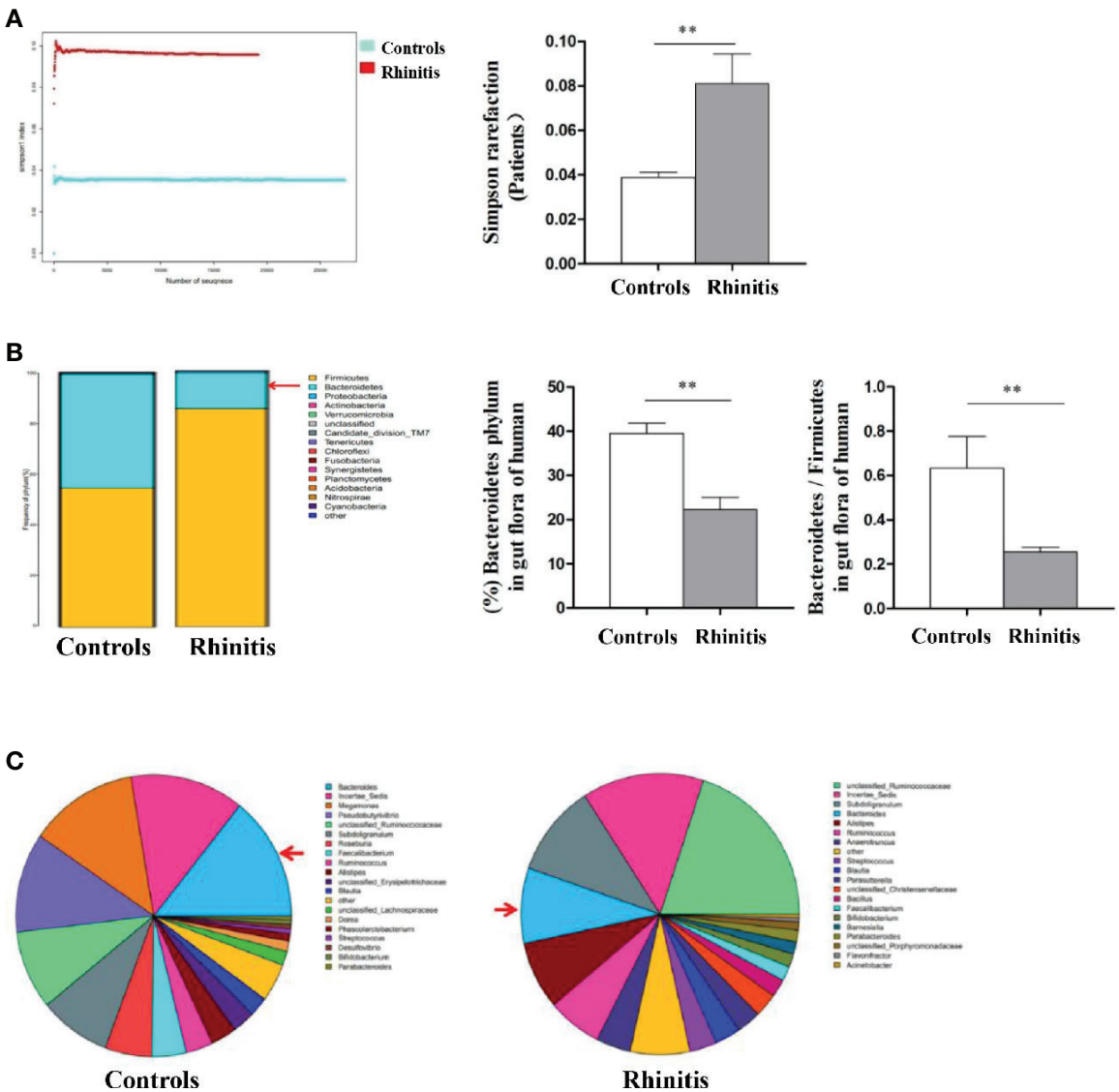


FIGURE 2 | The diversity and abundance of intestinal microflora in patients with allergic rhinitis. **(A)** The Simpson rarefaction in allergic rhinitis patients increased compared with that in healthy controls. **(B)** Bacterial composition and abundance at the phylum level. Red arrow: Bacteroidetes. **(C)** Bacterial composition and abundance at the genus level. Red arrow: Bacteroides. Bar graphs represent mean ± SD (n = 6). $^{**}P < 0.01$.

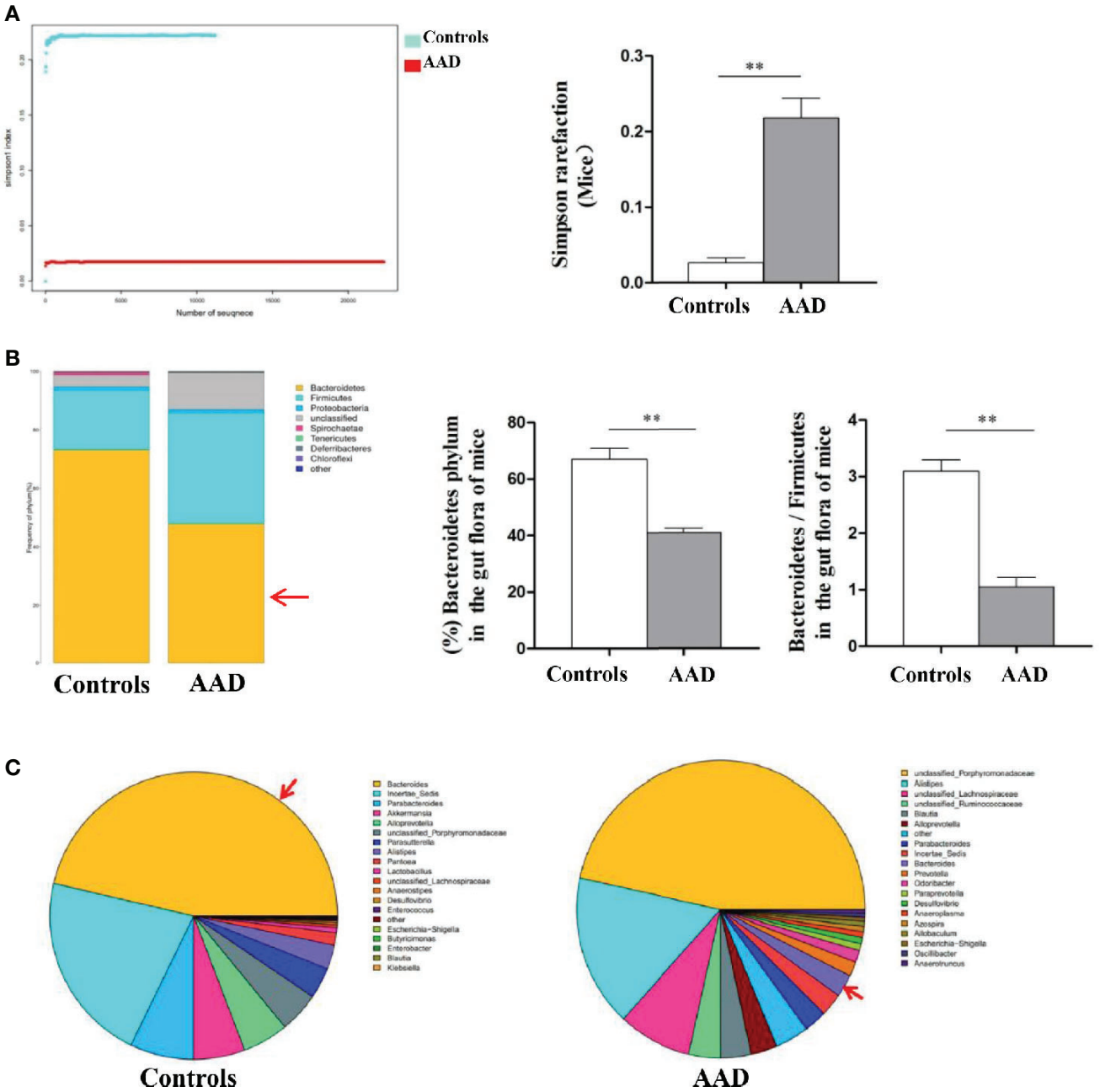


FIGURE 3 | The diversity and abundance of intestinal microflora in murine model of OVA-induced allergic airway inflammation. **(A)** The Simpson rarefaction in AAD mice increased. **(B)** Bacterial composition and abundance at the phylum level. Red arrow: Bacteroidetes. **(C)** Bacterial composition and abundance at the genus level. Red arrow: Bacteroides. Bar graphs represent mean ± SD (n = 6). $^{**}P < 0.01$.

airway inflammation. Taken together, enteric dysbiosis with the decrease of abundance of Bacteroides existed in allergic rhinitis patients and mice.

B. thetaiotaomicron Ameliorates AHR

B. thetaiotaomicron is one of the most abundant bacteria in gut flora, which belongs to the genus Bacteroides. We further tested *B. thetaiotaomicron* in OVA-induced allergic airway inflammation murine model system to explore the potential role of this bacteria in treating allergic airway inflammation (**Figure 1**). Firstly we measured the frequency of allergic symptoms. AHR was induced in mice of OVA group, resulting in an increased frequency of nasal rubbing (**Figure 4A**) and sneezing (**Figure 4B**). Interestingly, in the OVA/10^6BT and OVA/10^8BT groups the occurrences of nasal rubbing and sneezing were lower as compared with the occurrences in the OVA group; in the OVA/

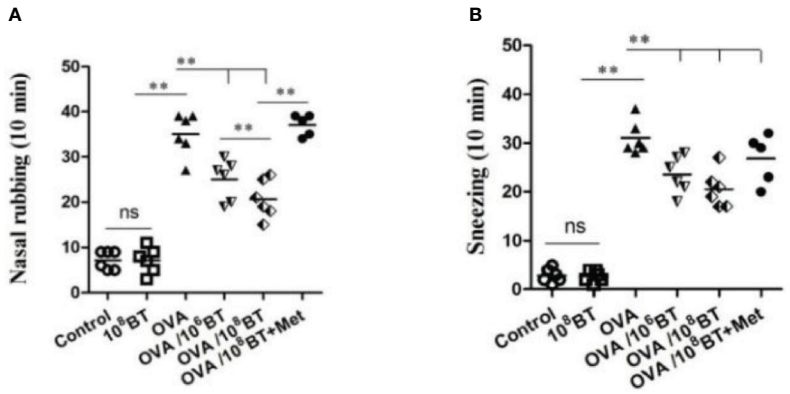


FIGURE 4 | The changes of allergic symptoms. OVA induced an obvious AHR in mice with increased frequency of nasal rubbing **(A)** and sneezing **(B)**. The occurrences of nasal rubbing and sneezing were noticeably reduced in OVA/BT group, while OVA/BT+Met inhibited the effect in ameliorating allergic symptoms. $^{**}P < 0.01$.

10^8BT+Met group metronidazole inhibited the effect in ameliorating allergic symptoms. The allergic symptoms between control and 10^8BT groups had no noteworthy differences (**Figure 4**).

B. thetaiotaomicron Attenuates OVA–Induced Airway Inflammation in Mice

We further examined the inflammatory cells from NALF and BALF samples (**Figure 5**). More inflammatory cells—eosinophils, neutrophils, monocytes, and lymphocytes—were detected in OVA group than the control group. Oral *B. thetaiotaomicron* administration before challenge in the OVA/10^6BT and OVA/10^8BT groups significantly reduced total cells and eosinophils in NALF and BALF. We also evaluated the mast cell and eosinophil infiltration in the nasal and lung by histological analysis to investigate the effect of oral *B. thetaiotaomicron* before challenge. There were no changes between the control and 10^8BT groups, while OVA group had mast cell (**Figure S1**) and eosinophil infiltration (**Figure S2**) in the nasal and lung. In the OVA/10^6BT and OVA/10^8BT groups, we found a significant suppression of mast cell and eosinophil infiltration by oral *B. thetaiotaomicron*. When metronidazole was co-administered with *B. thetaiotaomicron*, the suppression effect was weakened, and the airway inflammation was aggravated in OVA/10^8BT + Met group. These findings suggest that oral administration of *B. thetaiotaomicron* attenuates OVA-induced airway inflammation.

B. thetaiotaomicron Inhibits OVA-Induced Airway Th2 Cytokines and Serum OVA-Specific IgE

Levels of Th2 cytokines (IL-4, IL-5, and IL-13) in NALF and BALF samples of OVA group were significantly higher as compared with that of control group; OVA/10^6BT and OVA/10^8BT groups showed relatively lower Th2 cytokine levels as compared with OVA group (**Figure 6A**). When *B. thetaiotaomicron* was co-administered with metronidazole (OVA/10^8BT + Met group), the inhibitory effect of *B. thetaiotaomicron* on Th2 cytokine levels was weakened. However in BALF samples, levels of IL-10, the main cytokine secreted by regulatory T cells to mediate immune suppression, were significantly higher in OVA/10^6BT and OVA/10^8BT groups than OVA model group (**Figure 6B**). Also, levels of Th1 cytokine IFN-γ, were not significantly changed among the OVA, OVA/10^6BT, OVA/10^8BT, and OVA/10^8BT + Met groups in the lavage liquid (**Figure 6C**). Furthermore, oral administration of *B. thetaiotaomicron* in the OVA/10^6BT and OVA/10^8BT groups significantly reduced the production of OVA-induced serum IgE but not that of IgG1 (**Figure 6D**). The co-administration of metronidazole in OVA/10^8BT+Met group impaired the inhibition of IgE induced by *B. thetaiotaomicron*, similar to the findings of Th2 cytokines.

B. thetaiotaomicron Increases ICOS and IL-10 Expression in Treg Cells in the Mesenteric and Cervical Lymph Nodes (LNs) and Spleen

ICOS enhances the proliferation, survival, and function of Treg cells and has critical immunoregulatory implications (9). We investigated the effects of oral administration of *B. thetaiotaomicron* on the regulation of ICOS and Tregs in spleen, mesenteric LNs and cervical LNs that drains the nasal mucosa and further examined the frequency of $CD4^+$ T cells expressing either CD25 or ICOS, and Foxp3, as well as intracellular cytokine staining for IL-10 at specific time intervals. Ratios of $CD4^+Foxp3^+$ cells and $CD4^+ICOS^+$ cells were slightly increased in lymphocytes of the OVA model group as compared with that of the control group. When *B. thetaiotaomicron* was administered, ratios of $CD4^+Foxp3^+$ cells, $CD4^+ICOS^+$T cells, and $CD4^+ICOS^+Foxp3^+$ regulatory T cells were obviously upregulated in the lymphocytes of the spleen (**Figure S3**), mesenteric LNs (**Figure S4**) and cervical LNs (**Figure 7**) of OVA/10^8BT group as compared with that of the OVA model group. In addition, *B. thetaiotaomicron* increased

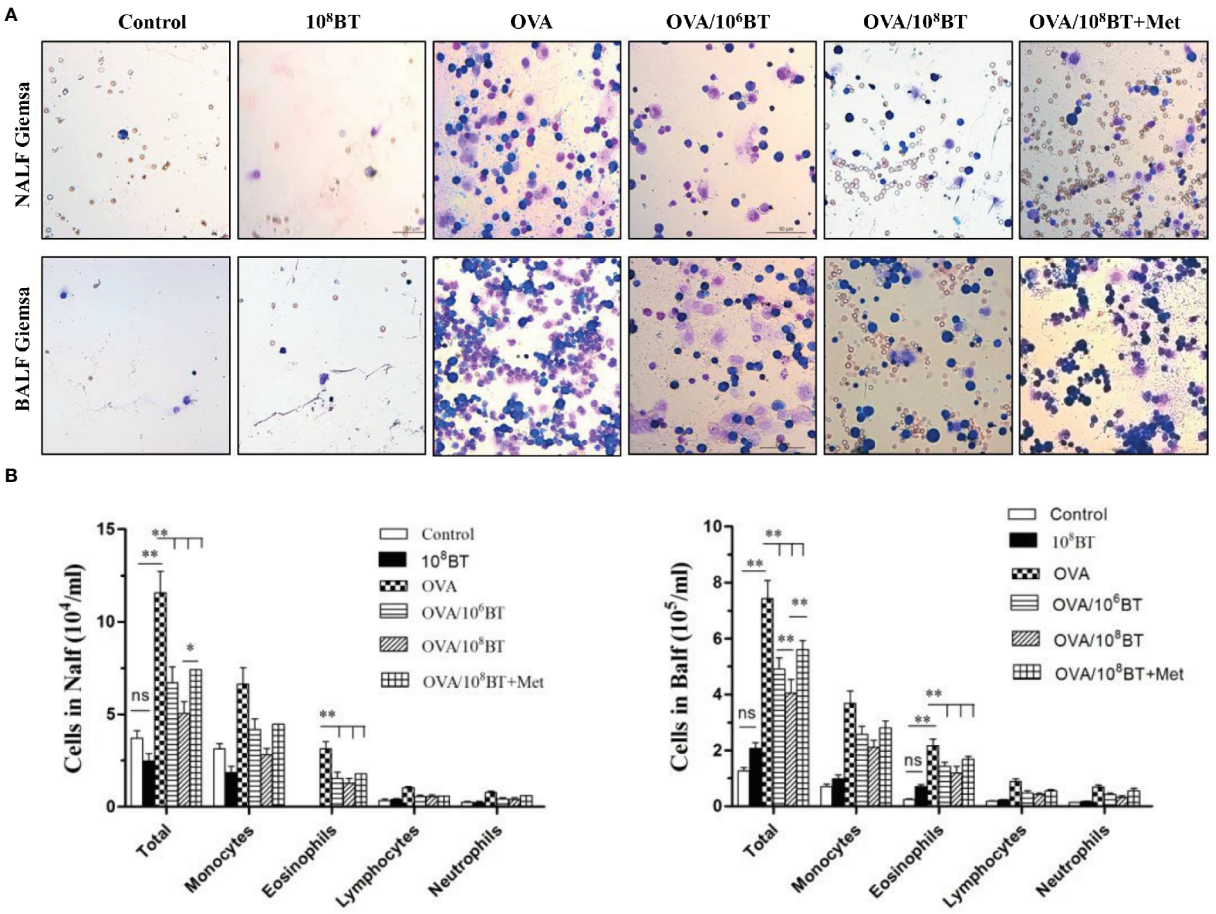


FIGURE 5 | OVA/BT inhibited OVA-induced airway inflammation. **(A)** Wright-Giemsa staining of inflammation cells in NALF and BALF at 24 h after final challenge. **(B)** Total inflammation cells and eosinophils in NALF and BALF. Bar graphs represent mean ± SD.*$P < 0.05$, **$P < 0.01$. ns, no significance.

ratio of IL-10-expressing $CD4^+Foxp3^+$ cells in the spleen (**Figure S3D**), mesenteric LNs (**Figure S4D**) and cervical LNs (**Figure 7D**) relative to OVA model group. These data indicated that application of *B. thetaiotaomicron* before allergen challenge was capable of establishing adequate Tregs to regulate immune system for preventing AHR *via* secretion of IL-10.

DISCUSSION

The healthy gut microbiota is a stable, diverse, resilient, and resistant microbial ecosystem. Firmicutes and Bacteroidetes are two major phyla, together representing ∼90% of the gut microbiota. Gut microbiota have a significant effect on systemic immunity and metabolism, which are involved in the occurrence and development of AAD (17–19). In children with allergic rhinitis and asthma, the abundance of phylum Firmicutes was lower as compared with that in healthy controls (20). However, we observed enteric dysbiosis with the decrease in biodiversity and lower abundance of phylum Bacteroidetes in both allergic rhinitis adult patients and mice. A plausible explanation for this variation can be that the gut microbiota coexists with the human body and changes with age. In the first 2–3 years of life, the gut microbiota varies extensively in composition and metabolic functions. After this period, the gut microbiota demonstrates adult-like more stable and diverse microbial species. However, in old age, the gut microbiota alters drastically and shows less diversity compared to younger age, which promotes various gut-related diseases. The number of gram negative bacteria increases during aging, which secretes lipopolysaccharide and causes inflammation in human gut (21, 22). A decrease of the Bacteroidetes to Firmicutes ratio was also observed in old compared to young mice (14).

B. thetaiotaomicron is one of the most efficient polysaccharide-degrading bacteria and contains exclusive polysaccharide-degrading enzymes not found in humans. *B. thetaiotaomicron* can produce up to 260 kinds of enzymes that can decompose starch, glycogen and cellulose into small molecules of glucose, thus helping the human body to effectively extract nutrients from wheat germ, vegetables, fruits and other foods. *B. thetaiotaomicron* can quickly adjust more than 25% of its genes to the active state according to the changes of food sources, so as to digest new nutrients and maintain the health

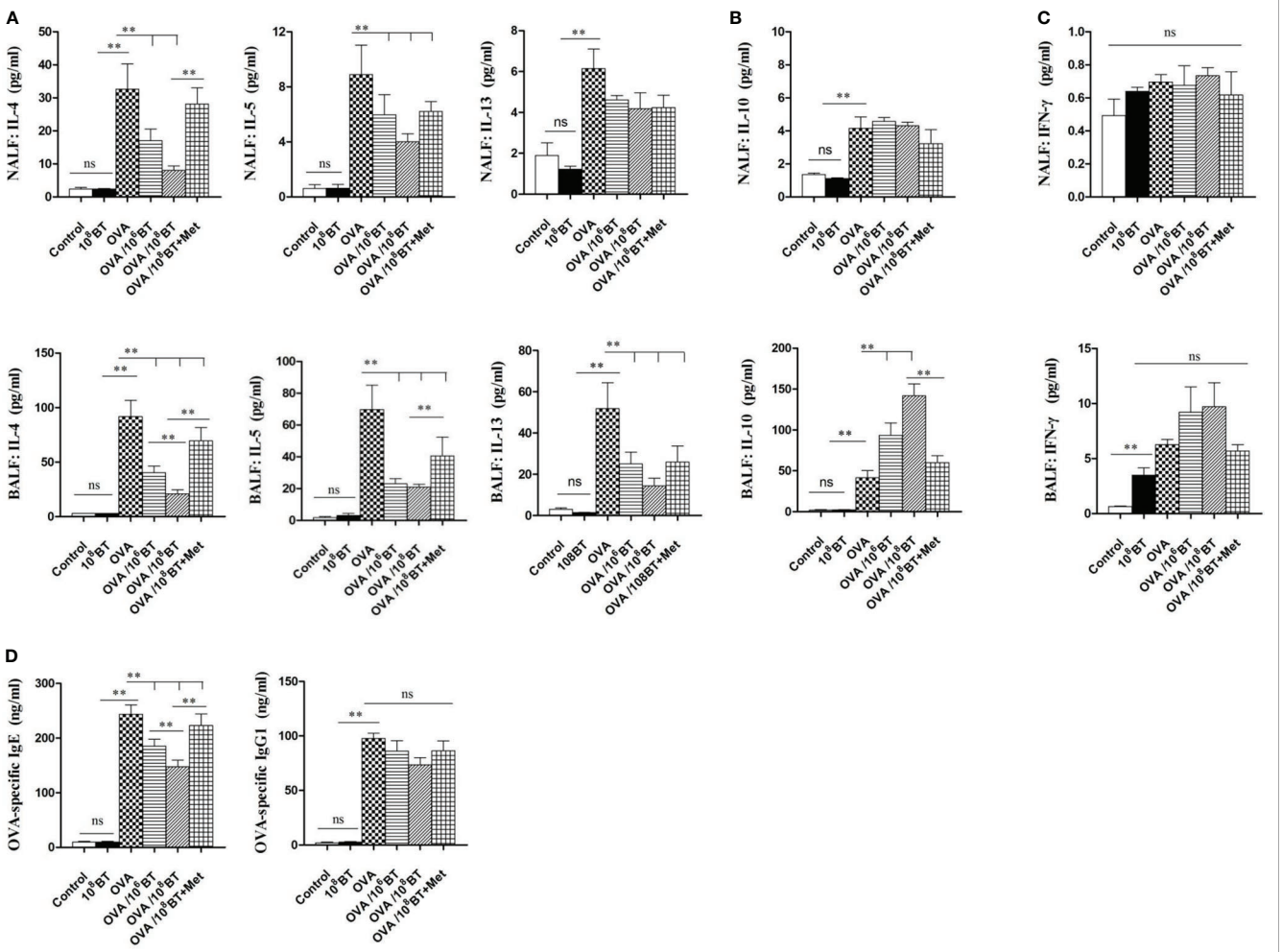


FIGURE 6 | The changes of cytokines IL-4, IL-5, IL-13 **(A)**, IL-10 **(B)** and IFN-γ **(C)** in NALF and BALF and serum OVA-specific IgE and IgG1 levels **(D)**. Oral *B. thetaiotaomicron* application inhibited secretion of Th2 cytokines IL-4, IL-5 and IL-13 and increased levels of IL-10 in BALF. Levels of Th1 cytokine IFN-γ in the lavage liquid were not significantly changed. Oral *B. thetaiotaomicron* application also reduced serum OVA-specific IgE production induced by OVA challenge significantly, but not IgG1 production.**$P < 0.01$. ns, no significance.

of the whole intestinal flora (23–25). The *B. thetaiotaomicron* ATCC 29148 strain is a standard strain originally purified from the feces of healthy adults (25). In the present study, we performed intervention assays using *B. thetaiotaomicron* ATCC 29148 (10^6CFUs and 10^8CFUs) and OVA-induced allergic airway inflammation murine model. The oral administration of ATCC 29148 in mice ameliorated the allergic symptoms of nose and reduced the frequency of scratching and sneezing. It also inhibited eosinophilic inflammation of nasal mucosa and bronchoalveolar tissues and the mast cell infiltration. The exudation of inflammatory cells, especially eosinophils, in the nasal cavity and alveolar lavage solution was also reduced. In addition, the expression levels of Th2 cytokines (IL-4, IL-5, and IL-13) and OVA-specific IgE were down-regulated. IL-4, IL-5, and IL-13 are known to regulate the growth and differentiation of eosinophils (26). Oral probiotics can alter the respiratory microbiota and have been advocated as a novel therapeutic strategy for AAD (27). Our findings are consistent with other reports demonstrating improvement in allergic rhinitis and asthma inflammation through oral administration of *Bifidobacterium breve* (28) and *Lactobacillus plantarum* (29). However, levels of the main cytokine secreted by Tregs to mediate immune suppression, IL-10, were significantly higher in ATCC 29148 intervention group as compared with the levels in control group. In addition, the level of Th1 cytokine, IFN-γ, was not influenced by *B. thetaiotaomicron* ATCC 29148, suggesting that ATCC 29148 influences Th1/Th2 equilibrium mainly through the inhibition of Th2 response without promoting a Th1 response. These observations can be explained by the characteristic differences between *B. thetaiotaomicron* and other probiotics. There are characteristic differences in growth, carbohydrate consumption or metabolite production among various species of bacteria. The immune responses induced by various species of bacteria are different. It has been shown that *Bifidobacterium adolescentis* did not challenge any production of bacteria specific serum antibodies

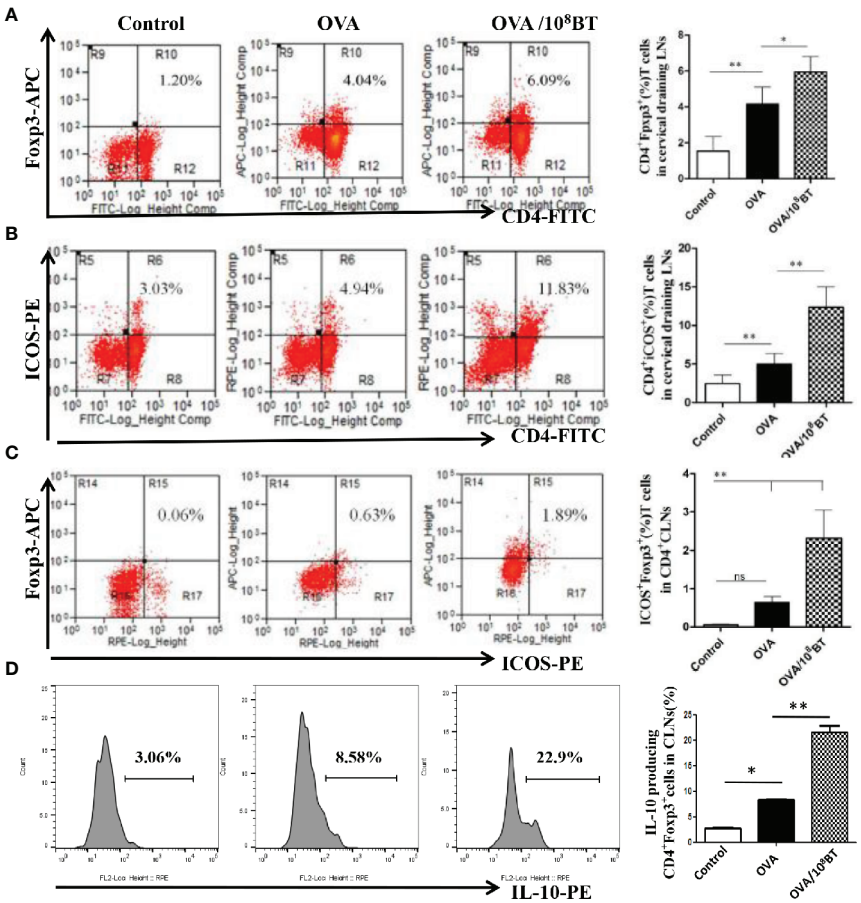


FIGURE 7 | *B. thetaiotaomicron* induced ICOS expression on Tregs and amplification of IL-10-expressing $CD4^+Foxp3^+$Tregs in draining cervical LNs. Representative scatter plots and ration of the fraction of $CD4^+Foxp3^+$ cells **(A)**, $CD4^+ICOS^+$T cells **(B)** and $CD4^+ICOS^+Foxp3^+$ regulatory T cells **(C)**. Representative histogram showing expression of IL-10 in $CD4^+Foxp3^+$ Tregs **(D)**. Bar graphs represent mean ± SD. n = 6, $^*P < 0.05$, $^{**}P < 0.01$. ns, no significance.

in comparison to germ-free rat, while *B. thetaiotaomicron* alone can induce a humoral response characterized by IgA and IgG production (30).

Interestingly, immunomodulatory effect of *B. thetaiotaomicron* ATCC 29148 was weakened and the allergic inflammatory response was aggravated, when ATCC 29148 was orally administered in mice in combination with metronidazole. These findings suggest that metronidazole can block the immunomodulatory effect of *B. thetaiotaomicron*. The overuse of antibiotics may result in strong selection pressure and influence the inherent flora of human intestinal tract, which is one of the reasons for the rapid increase of AAD incidence in recent years (31). McKeever et al. confirmed that the incidence of AAD is closely related with the use of antibiotics in infants (32). Therefore, antibiotics should only be used when needed, and their use should be regulated.

Tregs play an indispensable role in maintaining immune homeostasis and contribute to allergic disease management by suppressing Th2-type immune responses. The balance between Th2 and Tregs is crucial for the development or suppression of allergic airway inflammation (33). ICOS is expressed on antigen-primed T cells—activated effector T cells, Th2 cells, memory T cells, and Tregs—and plays a key role in T cell activation and differentiation (8, 9). Here, we analyzed the expression of ICOS on $CD4^+$ T cells and $Foxp3^+$ Tregs to investigate the immunomodulatory effect of *B. thetaiotaomicron* on allergic airway inflammation in mice. After oral administration of *B. thetaiotaomicron*, ratios of $CD4^+Foxp3^+$ cells, $CD4^+ICOS^+$ T cells, and $CD4^+ICOS^+Foxp3^+$ regulatory T cells were higher in the lymphocytes of spleen, mesenteric and cervical LNs of OVA/10^8BT groups compared with that of the OVA model group. *B. thetaiotaomicron* also increased ratio of IL-10-expressing $CD4^+Foxp3^+$ cells in the spleen, mesenteric LNs, and cervical LNs relative to OVA model group. These observations suggest that *B. thetaiotaomicron* intervention can induce the expansion of $CD4^+ICOS^+Foxp3^+$ and IL-$10^+CD4^+Foxp3^+$regulatory T cells, exerting an immunosuppressive effect on Th2 type response.

Collectively, our study demonstrated that enteric dysbiosis with the decrease of abundance of Bacteroides existed in AAD; oral administration of *B. thetaiotaomicron* significantly attenuates allergic airway inflammation in OVA-induced AAD model. *B. thetaiotaomicron* may open up novel possibilities in terms of therapeutic interventions for AAD. Alteration of the

ratio of *Firmicutes* to *Bacteroidetes* can directly affect the fiber metabolism by gut microbiota, consequently changing the concentration of circulating short-chain fatty acids (SCFAs). Production of various SCFAs meditated by gut microbiota has been shown to be important for host systemic immunity. SCFAs, especially butyrate, have exerted broad anti- inflammatory activities (34–36). It will be very interesting to explore the changes of metabolite production after alteration of the ratio of *Firmicutes* to *Bacteroidetes* in further studies on AAD.

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 authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Affiliated Hospital of Qingdao University Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by The Animal Care Committee of Qingdao University.

AUTHOR CONTRIBUTIONS

WP designed and performed most of the experiments. AL, MC, and LH provided help in some of the key experiments. YJ and JZ provided suggestions for the study. ZL and DW supervised the study, analyzed and interpreted 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.2021.620943/full#supplementary-material

Supplementary Figure 1 | Mast cell infiltration assessed on Toluidine Bluestained nasal mucosa and lung. **(A)** Mast cells were round or oval, with dark blue nucleus and purplish red or dark purple cytoplasm. Original magnification was ×200. **(B)** Number of mast cells in the nasal mucosa and lung. Bar graphs represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Supplementary Figure 2 | Eosinophil inflammation assessed on hematoxylin and eosin (HE) stained nasal mucosa and lung. **(A)** The eosinophils were round and the eosinophilic granules in cytoplasm were brick red or bright red. Original magnification was ×200. **(B)** Number of eosinophils in the nasal mucosa and inflammation score of lung. Bar graphs represent mean ± SD. $^{**}P < 0.01$.

Supplementary Figure 3 | *B. thetaiotaomicron* induced ICOS expression on Tregs and amplification of IL-10-expressing $CD4^{+}Foxp3^{+}$ Tregs in spleen. Representative scatter plots and ration of the fraction of $CD4^{+}Foxp3^{+}$ cells **(A)**, $CD4^{+}ICOS^{+}$T cells **(B)**, and $CD4^{+}ICOS^{+}Foxp3^{+}$ regulatory T cells **(C)**. Representative histogram showing expression of IL-10 in $CD4^{+}Foxp3^{+}$ Tregs **(D)**. Bar graphs represent mean ± SD. n = 6, $^{*}P < 0.05$, $^{**}P < 0.01$.

Supplementary Figure 4 | *B. thetaiotaomicron* induced ICOS expression on Tregs and amplification of IL-10-expressing $CD4^{+}Foxp3^{+}$ Tregs in mesenteric LNs. Representative scatter plots and ration of the fraction of $CD4^{+}Foxp3^{+}$ cells **(A)**, $CD4^{+}ICOS^{+}$T cells **(B)** and $CD4^{+}ICOS^{+}Foxp3^{+}$ regulatory T cells **(C)**. Representative histogram showing expression of IL-10 in $CD4^{+}Foxp3^{+}$ Tregs **(D)**. Bar graphs represent mean ± SD. n = 6, $^{*}P < 0.05$, $^{**}P < 0.01$.

REFERENCES

1. Brożek J, Bousquet J, Agache I, Agarwal A, Bachert C, Bosnic-Anticevich S, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines-2016 revision. *J Allergy Clin Immunol* (2017) 140:950–58. doi: 10.1016/j.jaci.2017.03.050
2. Platts-Mills T. The allergy epidemics: 1870-2010. *J Allergy Clin Immunol* (2015) 136:3–13. doi: 10.1016/j.jaci.2015.03.048
3. Zvezdin B, Hromis S, Kolarov V, Milutinov S, Zarić B, Jovancević L, et al. Allergic asthma and rhinitis comorbidity. *Vojnosanit Pregl* (2015) 72:1024–31. doi: 10.2298/vsp140605099z
4. Greiner A, Hellings P, Rotiroti G, Scadding G. Allergic rhinitis. *Lancet* (2011) 378:2112–22. doi: 10.1016/s0140-6736(11)60130-x
5. Takenaka MC, Quintana FJ. Tolerogenic dendritic cells. *Semin Immunopathol* (2017) 39:113–20. doi: 10.1007/s00281-016-0587-8
6. Wing JB, Tanaka A, Sakaguchi S. Human FOXP3(+) Regulatory T Cell Heterogeneity and Function in Autoimmunity and Cancer. *Immunity* (2019) 50:302–16. doi: 10.1016/j.immuni.2019.01.020
7. Zhou CJ, Ma F, Liao WJ, Song LJ, Yu D, Song YN, et al. Restoration of immune suppressor function of regulatory B cells collected from patients with allergic rhinitis with Chinese medical formula Yupingfeng San. *Am J Transl Res* (2019) 11:1635–43.
8. Dong C, Nurieva RI. Regulation of immune and autoimmune responses by ICOS. *J Autoimmun* (2003) 21:255–60. doi: 10.1016/s0896-8411(03)00119-7
9. Coyle AJ, Gutierrez-Ramos JC. The role of ICOS and other costimulatory molecules in allergy and asthma. *Springer Semin Immunopathol* (2004) 25:349–59. doi: 10.1007/s00281-003-0154-y
10. Kadkhoda K, Wang S, Fan Y, Qiu H, Basu S, Halayko AJ, et al. ICOS ligand expression is essential for allergic airway hyperresponsiveness. *Int Immunol* (2011) 23:239–49. doi: 10.1093/intimm/dxq476
11. Lynch SV. Gut Microbiota and Allergic Disease. New Insights. *Ann Am Thorac Soc* (2016) 13(Suppl 1):S51–4. doi: 10.1513/AnnalsATS.201507-451MG
12. Viennois E, Chassaing B. First victim, later aggressor: How the intestinal microbiota drives the pro-inflammatory effects of dietary emulsifiers? *Gut Microbes* (2018) 9:1–4. doi: 10.1080/19490976.2017.1421885
13. Noverr MC, Noggle RM, Toews GB, Huffnagle GB. Role of antibiotics and fungal microbiota in driving pulmonary allergic responses. *Infect Immun* (2004) 72:4996–5003. doi: 10.1128/IAI.72.9.4996-5003.2004
14. Vital M, Harkema JR, Rizzo M, Tiedje J, Brandenberger C. Alterations of the murine gut microbiome with age and allergic airway disease. *J Immunol Res* (2015) 2015:892568. doi: 10.1155/2015/892568
15. Pang W, Wang H, Shi L, Sun Y, Wang X, Wang M, et al. Immunomodulatory effects of Escherichia coli ATCC 25922 on allergic airway inflammation in a mouse model. *PLoS One* (2013) 8:e59174. doi: 10.1371/journal.pone.0059174
16. Kang JH, Kim BS, Uhm TG, Lee SH, Lee GR, Park CS, et al. Gamma-secretase inhibitor reduces allergic pulmonary inflammation by modulating Th1 and

Th2 responses. *Am J Respir Crit Care Med* (2009) 179:875–82. doi: 10.1164/rccm.200806-893OC
17. Samuelson D, Welsh D, Shellito J. Regulation of lung immunity and host defense by the intestinal microbiota. *Front Microbiol* (2015) 6:1085. doi: 10.3389/fmicb.2015.01085
18. Claassen-Weitz S, Wiysonge C, Machingaidze S, Thabane L, Horsnell W, Zar H, et al. Current Knowledge and Future Research Directions on Fecal Bacterial Patterns and Their Association with Asthma. *Front Microbiol* (2016) 7:838. doi: 10.3389/fmicb.2016.00838
19. Belkaid Y, Harrison OJ. Homeostatic Immunity and the Microbiota. *Immunity* (2017) 46:562–76. doi: 10.1016/j.immuni.2017.04.008
20. Chiu CY, Chan YL, Tsai MH, Wang CJ, Chiang MH, Chiu CC. Gut microbial dysbiosis is associated with allergen-specific IgE responses in young children with airway allergies. *World Allergy Organ J* (2019) 12:100021. doi: 10.1016/j.waojou.2019.100021
21. Schiffrin EJ, Morley JE, Donnet-Hughes A, Guigoz Y. The inflammatory status of the elderly: the intestinalcontribution. *Mutat Res* (2010) 690:50–6. doi: 10.1016/j.mrfmmm.2009.07.011
22. Kumar M, Babaei P, Ji B, Nielsen J. Human gut microbiota and healthy aging: Recent developments and future prospective. *Nutr Healthy Aging* (2016) 4:3–16. doi: 10.3233/NHA-150002
23. Reeves AR, D'Elia JN, Frias J, Salyers AA. A *Bacteroides thetaiotaomicron* outer membrane protein that is essential for utilization of maltooligosaccharides and starch. *J Bacterio* (1996) 178:823–30. doi: 10.1128/jb.178.3.823-830.1996
24. Reeves AR, Wang GR, Salyers AA. Characterization of four outer membrane proteins that play a role in utilization of starch by *Bacteroides thetaiotaomicron*. *J Bacteriol* (1997) 179:643–49. doi: 10.1128/jb.179.3.643-649.1997
25. Xu J, Bjursell MK, Himrod J, Deng S, Carmichael LK, Chiang HC, et al. A genomic view of the human-Bacteroides thetaiotaomicron symbiosis. *Science* (2003) 299:2074–76. doi: 10.1126/science.1080029
26. Cox L, Calderón M, Pfaar O. Subcutaneous allergen immunotherapy for allergic disease: examining efficacy, safety and cost-effectiveness of current and novel formulations. *Immunotherapy* (2012) 4:601–16. doi: 10.2217/imt.12.36
27. Vientós-Plotts A, Ericsson A, Rindt H, Reinero C. Oral Probiotics Alter Healthy Feline Respiratory Microbiota. *Front Microbiol* (2017) 8:1287. doi: 10.3389/fmicb.2017.01287
28. Ren J, Zhao Y, Huang S, Lv D, Yang F, Lou L, et al. Immunomodulatory effect of *Bifidobacterium breve*on experimental allergic rhinitis in BALB/c mice. *Exp Ther Med* (2018) 16:3996–4004. doi: 10.3892/etm.2018.6704
29. Choi S, Oh H, Choi C, Ahn H, Yun H, Chung Y, et al. Oral administration of *Lactobacillus plantarum* CJLP133 and CJLP243 alleviates birch pollen-induced allergic rhinitis in mice. *J Appl Microbiol* (2018) 124:821–28. doi: 10.1111/jam.13635
30. Scharek L, Hartmann L, Heinevetter L, Blaut M. Bifidobacterium adolescentis modulates the specific immune response to another human gut bacterium, Bacteroides thetaiotaomicron, in gnotobiotic rats. *Immunobiology* (2000) 202:429–41. doi: 10.1016/s0171-2985(00)80102-3
31. Cullinan P, Harris J, Mills P, Moffat S, White C, Figg J, et al. Early prescriptions of antibiotics and the risk of allergic disease in adults: a cohort study. *Thorax* (2004) 59:11–5. doi: 10.1136/thx.2003.019877
32. McKeever TM, Lewis SA, Smith C, Collins J, Heatlie H, Frischer M, et al. Early exposure to infections and antibiotics and the incidence of allergic disease: a birth cohort study with the West Midlands General Practice Research Database. *J Allergy Clin Immunol* (2002) 109:43–50. doi: 10.1067/mai.2002.121016
33. Arnold IC, Dehzad N, Reuter S, Martin H, Becher B, Taube C, et al. Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. *J Clin Invest* (2011) 121:3088–93. doi: 10.1172/jci45041
34. Meijer K, deVos P, Priebe MG. Butyrate and other short-chain fatty acids as modulators of immunity: what relevance for health? *Curr Opin Clin Nutr Metab Care* (2010) 13:715–21. doi: 10.1097/MCO.0b013e32833eebe5
35. denBesten G, vanEunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res* (2013) 54:2325–40. doi: 10.1194/jlr.R036012
36. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med* (2014) 20:159–66. doi: 10.1038/nm.3444

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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Deposition of Immune Complexes in Gingival Tissues in the Presence of Periodontitis and Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is a complex chronic autoimmune disease characterized by tissue damage and widespread inflammation in response to environmental challenges. Deposition of immune complexes in kidneys glomeruli are associated with lupus nephritis, determining SLE diagnosis. Periodontitis is a chronic inflammatory disease characterized by clinical attachment and bone loss, caused by a microbial challenge – host response interaction. Deposition of immune complex at gingival tissues is a common finding in the course of the disease. Considering that, the primary aim of this study is to investigate the deposition of immune complexes at gingival tissues of SLE patients compared to systemically healthy ones, correlating it to periodontal and systemic parameters. Twenty-five women diagnosed with SLE (SLE+) and 25 age-matched systemically healthy (SLE–) women were included in the study. Detailed information on overall patient's health were obtained from file records. Participants were screened for probing depth (PD), clinical attachment loss (CAL), gingival recession (REC), full-mouth bleeding score (FMBS) and plaque scores (FMPS). Bone loss was determined at panoramic X-ray images as the distance from cementenamel junction to alveolar crest (CEJ-AC). Gingival biopsies were obtained from the first 15 patients submitted to surgical periodontal therapy of each group, and were analyzed by optical microscopy and direct immunofluorescence to investigate the deposition of antigen-antibody complexes. Eleven (44%) patients were diagnosed with active SLE (SLE-A) and 14 (56%) with inactive SLE (LES-I). Mean PD, CAL and FMBS were significantly lower in SLE+ than SLE–($p < 0.05$; Mann Whitney). The chronic use of low doses of immunosuppressants was associated with lower prevalence of CAL >3 mm. Immunofluorescence staining of markers of lupus nephritis and/or proteinuria was significantly increased in SLE+ compared to SLE–, even in the presence of

periodontitis. These findings suggest that immunomodulatory drugs in SLE improves periodontal parameters. The greater deposition of antigen-antibody complexes in the gingival tissues of patients diagnosed with SLE may be a marker of disease activity, possibly complementing their diagnosis.

Keywords: systemic lupus erythematosus, periodontitis, immune complex, inflammation, diagnosis

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic, autoimmune disease caused by a combination of genetic, environmental and immunologic factors. It is characterized by varying clinical manifestations and by the presence of several autoantibodies, including anti-DNA, anti-nuclear (ANA) or anti-phospholipids antibodies. It affects mostly women at 15–45 years, in a proportion of 10:1 (1, 2). Its incidence is relatively rare, affecting 56 out of 100,000 Americans (3) and 87 out of 100,000 in Brazil (4). Approximately, 1.5 million Americans and 5 million people around the world have lupus (5).

The diagnosis of SLE is complex, since it presents periods of flare and quiescence and a wide range of clinical manifestations, such as malar rash, pain, fatigue, hair loss, physical impairment, seizures or psychosis, anemia, leukopenia or lymphopenia. The American College of Rheumatology (6, 7) and the Systemic Lupus International Collaborating Clinics (SLICC) (8) have defined that $\geq$4 criteria must be present to determine SLE diagnosis. SLICC classification criteria requires that one clinical and one laboratory criteria must be present or biopsy-proven lupus nephritis with positive ANA or anti-dsDNA antibodies. Diagnosis cannot be made at the onset of symptoms, and long periods of observation are necessary up to new clinical manifestations determine SLE. Immunological findings include depression of complement (low C3 and/or C4 or CH50) and high titers of varying circulating autoantibodies and deposition of immune complexes capable of activating complement and inflammation, resulting in multiorgan damage (9). Recently, a scoring system based on positive history of ANA through Hep-2 immunofluorescence was proposed (10). The production of anti-dsDNA is also considered as a cardinal sign of lupus, as its levels are correlated with disease activity (11).

Lupus nephritis (LN) is characterized by immune aggregates at sites of injury in glomeruli and in the tubules in $\sim$ 2/3 of renal biopsies. These immune complexes may be derived from circulating complexes or from *in situ* combination of antigen and antibody. Usually, patients with lupus nephritis show antibodies against dsDNA, Sm and C1q (12). In clinical practice, it is essential to evaluate patients' kidney status. A renal biopsy is a standard diagnostic tool for the evaluation of kidney lesions in SLE, but due to its invasive nature, a kidney biopsy has potential risks and as a rule, it is not routinely performed (13).

Not all SLE patients develop LN. It is significantly more prevalent especially in blacks, associated to genetic risk factors. LN occurs when the expression of neutrophil-associated genes increases, preceded by upregulation of proinflammatory transcripts. Even after death, neutrophils may contribute to tissue damage through the formation of neutrophils extracellular traps (NETS) that may facilitate inflammation and cause endothelial damage, amplifying autoimmunity (14).

Periodontitis is a chronic infectious and inflammatory disease caused by microbial dysbiosis, characterized by loss of attachment and alveolar bone around natural teeth. Secondary features include pocket formation, bleeding on probing, tooth mobility and suppuration, among others. Histopathological findings of advanced periodontal lesions depicts plasma cells and lymphocytes occupying a vast area of gingival connective tissues and elevated serum titers of IgG against periodontal pathogens, resulting in the formation of immune complex that deposit on gingival tissues (15–20), activating complement and neutrophils, and triggering inflammatory responses (21). Worse severity of periodontitis is associated to an exacerbated inflammatory response to microbial challenge.

The pathogenic mechanisms of periodontitis and SLE presents some similarities, as with other autoimmune diseases (22, 23). Deregulation of immune system, with a key role exerted by neutrophils, phagocytic cells and pro-inflammatory cytokines contribute to tissue destruction in both conditions (24). Recently, our research group showed significant upregulation of serum proinflammatory cytokines in individuals with SLE compared to controls. Anti-inflammatory IL4 and IL-10 were upregulated only in inactive SLE sera, controlling clinical phenotypes. Out of 24 oral microbial abundances, 14 unique subgingival bacteria profiles were elevated at SLE, especially *T. denticola* and *T. forsythia* in active SLE compared to inactive SLE and healthy controls. These findings suggested that low-grade systemic inflammation that influence SLE activity and severity are correlated to dysbiotic changes of the oral microbiota in periodontitis patients (25).

The prevalence, incidence and severity of periodontitis in SLE patients are controversial. Some studies show similar or better conditions in SLE compared to systemically healthy patients (26–28), while others suggested worst periodontal conditions in SLE (29–32). These conflicting results indicate that further research is necessary to better investigate the association between SLE and periodontitis.

The relevance and innovation of this study is that it investigates the presence of autoantibodies related to SLE in gingival tissue, which could contribute to the diagnosis of SLE. As a secondary goal, we aimed at investigating the prevalence, extension and severity of periodontitis in SLE patients compared to systemically healthy ones.

MATERIALS AND METHODS

Subjects

SLE subjects were recruited at Lauro de Souza Lima Hospital and Bauru State Hospital from November 2017 to July 2019. It were included in the test group (SLE+) 25 female 20–65 years diagnosed with SLE according to ACR 1982/1997 revised classification criteria by a rheumatologist specialist. All patients were, at the moment of inclusion, in monitoring and/or treatment of SLE at one of the two Hospitals where the study was carried on. Control group (SLE–) was composed by 25 systemically healthy age-matched women, recruited at the Clinics of Periodontics at School of Dentistry at Bauru-USP during the same time period. It were excluded from the study patients with other autoimmune and/or rheumatological disorders (e.g., rheumatoid arthritis, Sjögren syndrome, pemphigoid, lichen planus), diabetics, pregnant women, presence of fixed orthodontic appliances, use of antibiotics in the 6-month period previous to inclusion, previous periodontal treatment (<12 mo.), edentulous, <8 teeth remaining, chronic renal failure requiring dialysis or diagnosis of malignant neoplasms <5 years.

SLE Status

SLE diagnosis followed the guidelines defined by revised American College of Rheumatology criteria (6, 7). SLE activity was investigated by Systemic Lupus Erythemathosus Disease Activity Index (SLEDAI) (33). SLE inactivity was defined by SLEDAI ≤2 or by stable medications dose for at least 3 months and/or by daily prednisone dose <10 mg (8, 34). SLE activity was defined by SLEDAI >2 or daily prednisone doses >10 mg (8, 35). Disease severity was measured according to SLICC/ACR-DI (Systemic Lupus International Collaborating Clinics of American College of Rheumatology Damage Index) (35) to further characterize the studied population. SLE patients were subdivided into two groups based on the disease activity: active (SLE-A) or inactive (SLE-I). Antibodies anti-dsDNA were detected by immunofluorescence by the use of *Crithida luciliae* as substrate.

Clinical Examinations

All participants have answered a health questionnaire to investigate medical and dental history. A visual examination of oral cavity was performed with the aid of disposable spatulas to investigate lesions at internal and external portions of lower and upper lips, cheek mucosa, retromolar area, tongue, oral floor, hard and soft palate, isthmus of the faces and upper and lower gingival mucosas.

Clinical periodontal examination was performed by a single trained examiner (JRP) by using a UNC-15 millimeter periodontal probe at six sites/tooth according to: pocket probing depth (PD), clinical attachment loss (CAL) and full-mouth bleeding scores (FMBS). Gingival recession (REC) was also determined at six sites/tooth as the distance from cementum-enamel junction (CEJ) to gingival margin. Full mouth plaque scores (FMPS) were assayed in four sites/tooth after visual inspection. Bone loss was determined in extra-oral digital panoramic X-ray images (1:1) as the distance from CEJ to alveolar bone crest (CEJ-AC) at mesial and distal sites of the tooth with the worst clinical periodontal condition (36).

All participants underwent non-surgical periodontal therapy before biopsy collection. Treatment consisted of supra and subgingival scaling and root planing, dental prophylaxis, oral hygiene instruction, elimination of plaque retention factors (open cavities, overhanging restorations, hopeless teeth), control of traumatogenic forces and splinting of mobile teeth.

Histopathological and Direct Immunofluorescence Analysis

Incisional biopsies were obtained from gingival margin of 15 LES+ and 15 LES– patients requiring surgical periodontal treatment or for differential diagnosis of desquamative gingivitis lesions. All surgeries were performed by a single trained operator (JRP) under local anesthesia at Lauro de Souza Lima Hospital Dental Clinics (LES+) or at the Clinics of Periodontics, School of Dentistry at Bauru (LES–Samples were kept in cold saline solution for transfer to the laboratory, included in freezing medium, wrapped in aluminum foil and stored at −80°C until processing, which was performed at the Pathology Laboratory of Lauro de Souza Lima Hospital (Bauru, Brazil) by two pathologists (AJFN and MRSN).

Fragments were unfreeze and included in in glycolic resin embedding medium for cryotomy (Tissue-Tek, Sakura Finetek Europe BV, Netherland). Sections 5 μm-thick were obtained by using a cryostate (Leica CM1850, Leica Biosystems, Buffalo Grove, USA) at −22°C. Seven slides were obtained from each sample, each one containing two sections.

One section was stained by hematoxilin-eosin for descriptive histopathological analysis. The other section was prepared for direct immunofluorescence assay. Sections were delimited with a thin trace of varnish. After that, 100 μL of rabbit polyclonal FITC-conjugated antibodies (BioSB, Santa Barbara, CA, USA) against IgM, IgG, IgA, C1q, C3c diluted at 1:20 and F1b diluted at 1:80 were pipetted on the sections. Samples were incubated in humid and dark chamber for 30 min at 37°C, followed by washing in PBS and mounted in glass coverslips with 0.1% Evans Blue glycerin solution. Slides were wrapped in aluminum fail and kept at −20°C until the beginning of immunofluorescence reaction. After that, slides were kept in refrigerator at 4°C until analysis by fluorescence microscope (Zeiss Axioplan 2, Carl Zeis Microscopy, São Paulo, Brazil). The immunofluorescence reaction was classified semiquantitatively according to the intensity, varying from 1 to 3 (+, ++, +++).

Statistical Analysis

Statistical analysis was performed at GraphPad Prism 8.0 for Mac software, at a 5% significance level for all analysis. Comparisons between groups were performed by Mann Whitney since a non-normal distribution was observed by Kolmogorov-Smirnov test. The association between systemic and oral conditions was investigated by Chi-square test. Histopathological findings were qualitatively described. The results from direct immunofluorescence were analyzed by Mann Whitney and Fischer exact test.

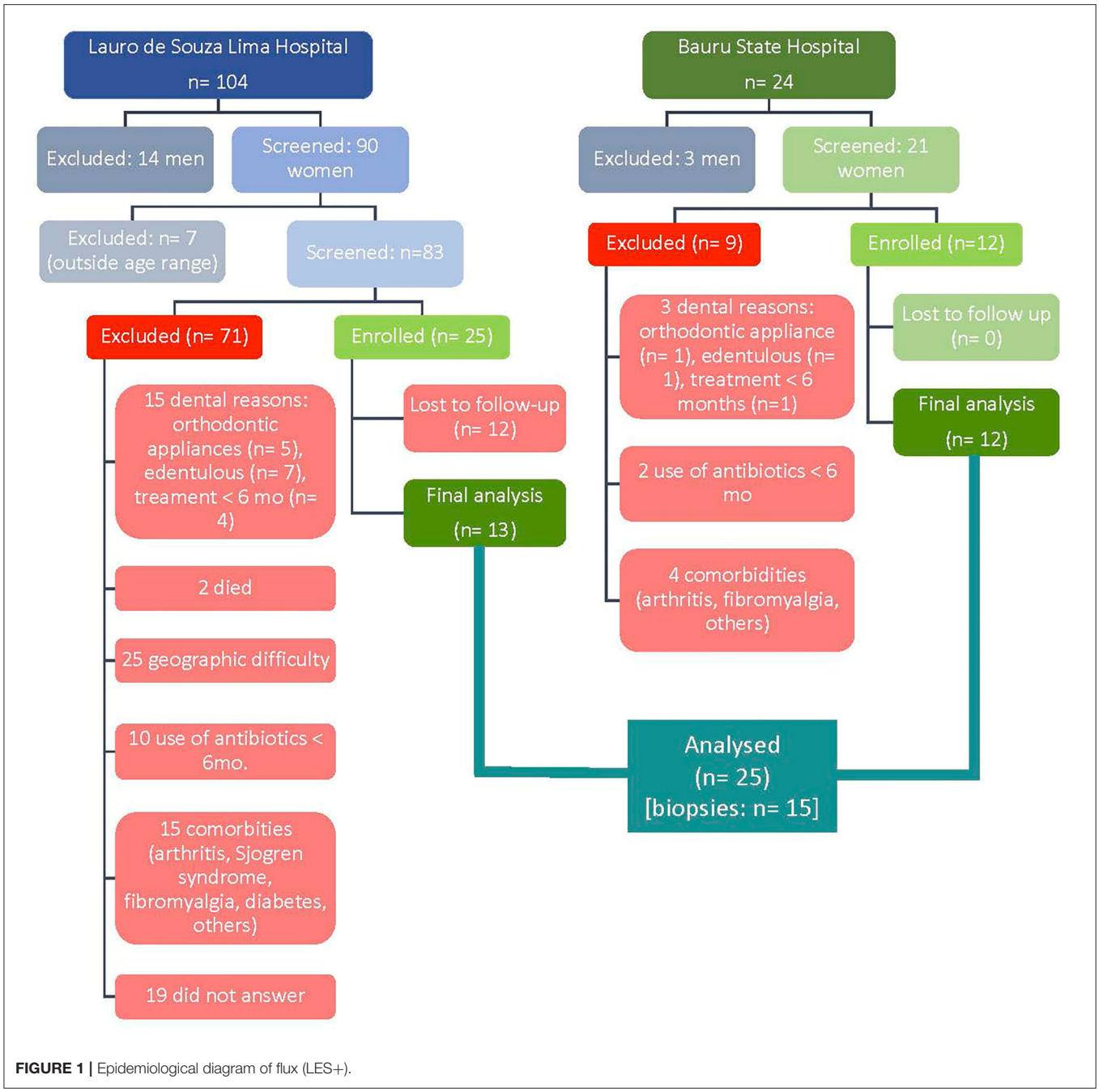

FIGURE 1 | Epidemiological diagram of flux (LES+).

RESULTS

A total of 104 medical records of women diagnosed with SLE at the Medical and Statistical Archive Service of Lauro de Souza Lima Hospital were screened for inclusion in this study. From that, 13 women showing SLE only were included. The remaining 12 women included in test group were recruited at Bauru State Hospital, according to the same inclusion/exclusion criteria. Participants of the control group gender- and age-matched were recruited at School of Dentistry at Bauru and consecutively included in the study (**Figure 1**). The overall characteristics of SLE+ and SLE− groups are described in **Table 1**.

Characteristics of Active and Inactive SLE

Eleven patients (44%) had active (SLE-A) and 14 inactive (SLE-I) lupus. Characteristics of the groups are described in **Table 2**. No respiratory disorders were observed at SLE-A, which maximum daily dose of prednisone was higher than SLE-I to control flare. SLE-A showed greater circulating titers of anti-dsDNA and lower C4. The frequency of disease active descriptors in both groups is presented as **Supplementary Table 1**.

TABLE 1 | Overall characteristicas of the sample.

	SLE+ (*n* = 25)	SLE– (*n* = 25)	*p*-value
Age			
Age [mean (sd)]	41.34 (12.39)	43.73 (14.04)	0.53**
Body mass index			
BMI [mean (sd)]	27.46 (5.95)	26.60 (5.32)	0.58**
Race			
Caucasians [*n* (%)]	16 (64)	22 (88)	0.08+
Brown [*n* (%)]	6 (24)	3 (12)	
Black [*n* (%)]	3 (12)	0 (0)	
Smoking			
Smoker (*n* %)	3 (12)	0 (0)	0.20+
Former smoker (*n* %)	4 (16)	5 (20)	
Non-smoker (*n* %)	18 (72)	20 (80)	
Medications			
Oral contraceptives (*n* %)	4 (16)	6 (24)	0.47+
Antihypertensive❖ (*n* %)	2 (8)	4 (16)	0.38+

***T-test; significant if p < 0.05; +Chi-square; significant if p < 0.05; ❖Hydrochlorothiazide; sd- standard deviation; n-absolute number; %–percentage.*

Periodontal Parameters

Mean PD, CAL and FMBS were significantly greater in SLE– women than in SLE+, with no significant differences observed in FMPS, CEJ-AC and tooth loss. Additionally, a greater percentage of sites with PD ≥5 mm was observed in SLE– (**Table 3**). Periodontitis was diagnosed in 22 (88%) of SLE+ and in 20 (80%) of SLE– participants, with no differences between groups. There was a prevalence of Stage III periodontitis, affecting 17 (68%) and 18 (72%) of SLE+ and SLE–, respectively. Most patients were assigned as Grade B, with no differences between SLE+ (52%) and SLE– (44%). Additionally, extension of periodontitis lesion was <30% (localized) in 86.36% of SLE-A and in 60% of SLE– participants. More detail in **Supplementary Table 2**.

The association between periodontal parameters and systemic conditions was investigate by Chi-squared bivariate analysis. CAL was significantly lower in patients using low daily doses of prednisone (≤10 mg/day), suggesting a beneficial effect of the chronic use of immunosuppressants in periodontal attachment loss (**Table 4**). Details on the characteristics of drug therapy for SLE+ are presented in **Supplementary Table 3**.

Histopathological Analysis

Gingival biopsies were obtained from 13 SLE+ diagnosed with periodontitis, 2 SLE+ with no periodontitis, and 15 SLE– with periodontitis. Histopatologic findings from SLE+ showed minimal mononuclear inflammatory infiltrate in the chorion, predominantly perivascular, with no significant morphological changes in the epithelium and intact basement membrane (**Figures 2A–D**). Histologic characteristics of LES- showed a dense inflammatory infiltrate predominantly composed by lymphocytes and plasma cells, occupying a vast area of gingival connective tissue (**Figures 2E–H**).

TABLE 2 | Overall characteristics of LES-A and LES-I.

	LES-A	LES-I	Valor do p
Age (mean ± sd)	38.18 ± 12.26	43.82 ± 12.36	0.31**
Gender: female (*n* %)	11 (100%)	14 (100%)	–
Ethnicity [*n* (%)]			0.23+
White	5 (45.45)	11 (78.58)	
Mixed	4 (36.36)	2 (28.58)	
Black	2 (18.19)	1 (7.14)	
Central nervous system disease (*n* %)	2/8 (25)	0/5 (0)	0.15+
Kidney disease (*n* %)	5/8 (62.5)	3/7 (42.86)	0.44+
Respiratory disorders (*n* %)	0/8 (0)	3/7 (42.86)	**0.03+**
Cardiovascular diseases (*n* %)	4/8 (50)	3/7 (42.86)	0.78+
Gastoenteric disease (*n* %)	1/8 (12.50)	1/7 (14.29)	0.91+
Comorbiditis (*n* %)	4/11 (36.36)	10/14 (71.43)	0.07+
Overweight or obesity (IMC ≥25)			
Hipertension	4/8 (50)	3/7 (42.86)	0.78+
Smoking (smokers and former smokers)	2/11 (18.18)	5/14 (35.71)	0.33+
Medications in use Prednisone dosage (mg/day) [mean ± sd]	18.64 ± 17.76	3.57 ± 3.63	**0.049****
Hidroxicloroquine (*n* %)	11/11 (100)	11/14 (78.57)	0.10+
Antihypertensive (*n* %)	2/11 (18.18)	6/14 (42.86)	0.18+
Antidepressants, anxyolitics (*n* %)	1/11 (9.09)	4/14 (28.57)	0.22+
Imunossupressants (*n* %)	1/11 (9.09)	4/14 (28.57)	0.22+
Chemotherapy (*n* %)	2/11 (18.18)	1/14 (7.14)	0.39+
Immuno-inflammatory response	5/8 (62.5)	0/5 (0)	**0.02+**
High anti-dsDNA titers (*n* %)			
Low C4 (*n* %)	5/8 (62.5)	0/5 (0)	**0.02+**
Low C3 (*n* %)	3/8 (37.5)	1/5 (20)	0.50+
SLEDAI (mean ± sd)	11.0 ± 8.42	0.4 ± 0.89	**0.005****

*Medical history and laboratory tests are not available for the entire sample; available data (number of positive cases / total cases (%); **t-test; +Chi-Square Test; significant if p < 0.05; sd, standard deviation. Bold values - significant differences between groups.*

Direct Immunofluorescence

Direct immunofluorescence was performed on all biopsies (*n* = 15/group) with anti-IgG, anti-IgM, anti-IgA, anti-C1q, anti-C3c (LN markers) and anti-fibrinogen (F1b) FITC-conjugated antibodies. SLE + patients demonstrated immunopositivity for IgG (**Figures 3A–D**) and IgM (**Figures 3E–H**) antibodies, mainly in the loose connective tissue regions permeating the epithelial ridges. Fibrinogen was homogeneously expressed in the connective tissue of SLE+ patients (**Figures 3I–L**). Additionally, SLE+ showed immunopositivity for IgA and C3c. The anti-C1q antibody was not reactive to immunofluorescence in any patient of both groups. In SLE, immune complexes IgG, IgM, IgA, and C3c were directed against basement membrane.

Quantitative analysis of direct immunofluorescence is shown in **Table 5**. Only 1 SLE– patient showed the

TABLE 3 | Periodontal parameters observed in SLE+ and SLE– women.

	SLE+	SLE–	*p*-value
REC			
mean (sd)	0.15 (0.19)	0.22 (0.39)	0.41*
median (95% CI)	0.11 (0.07; 0.23)	0.05 (0.06; 0.39)	
PD			
mean (sd)	2.18 (0.55)	2.87 (1.06)	**0.01***
median (95% CI)	2.06 (1.95; 2.41)	2.67 (2.44; 3.31)	
CAL			
mean (sd)	2.34 (0.53)	3.07 (1.14)	**0.005***
median (95% CI)	2.19 (2.12; 2.56)	2.79 (2.60; 3.54)	
FMBS			
mean (sd)	26.86 (16.14)	49.53 (33.78)	**0.01***
median (95% CI)	23.89 (20.19; 33.52)	50 (35.59; 63.48)	
FMPS			
mean (sd)	49.53 (22.16)	0.53 (0.35)	0.46*
median (95% CI)	50 (40.39; 58.68)	61.46 (39.35; 68.53)	
CEJ-AC			
mean (sd)	1.37 (0.68)	1.23 (0.52)	0.26*
median (95% CI)	1.24 (1.08; 1.64)	1.14 (1.02; 1.45)	
Missing teeth			
mean (sd)	7.68 (5.44)	8.28 (5.18)	0.69*
median (95% CI)	6 (5.43; 9.92)	6 (6.14; 10.42)	
% sites PD ≥5 mm			
median (95%CI)	1.23 (1.51; 6.35)	6.94 (6.97; 19.95)	**0.002***
% sites CAL ≥4 mm			
median (95%CI)	7.14 (7.99; 16.74)	22.22 (17.36; 35.69)	0.06*

**Mann Whitney; significante se p < 0.05. Bold values - significant differences between groups.*

TABLE 4 | Association between daily doses of prednisone and periodontal parameters.

	>10 mg/day	≤ 10 mg/day	*p* value	OR (95% CI)
PD > 2 mm	3 (12%)	9 (36%)	0.91	1.11 (0.21; 5.72)
PD ≤ 2 mm	3 (12%)	10 (40%)		
CAL > 3 mm	2 (8%)	1 (4%)	**0.03**	12.67 (1.03; 189.6)
CAL ≤ 3 mm	3 (12%)	19 (76%)		
FMBS ≥ 30%	1 (4%)	7 (28%)	0.74	0.66 (0.04; 5.30)
FMBS < 30%	3 (12%)	14 (56%)		
CEJ-ABC > 2	1 (4%)	1 (4%)	0.36	3.6 (0.15 – 71.43)
CEJ- ABC ≤ 2	5 (20%)	18 (72%)		

Chi-square test; significant if p < 0.05. Bold values - significant differences between groups.

presence of LN markers in gingival biopsies, while 9 SLE+ showed LN markers ($p = 0.005$; OR = 21; 2.46; 242.9; Fischer Exact Test). Comparisons between groups showed significant more labeling of IgM in SLE+ than SLE–. Both groups showed antibodies anti-F1q in the connective tissue. Detailed information is presented in **Supplementary Table 4**.

DISCUSSION

The diagnosis of SLE is complex and relies on the presence of four signs and symptoms, necessarily including one clinical and one laboratorial finding or the biopsy-proven lupus nephritis (LN) (1). We have used antibodies against IgM, IgG, IgA, C1q and C3c, frequently used for the diagnosis of LN by direct immunofluorescence, to investigate the deposition of immune complexes in the gingival connective tissue in SLE patients compared to systemically healthy ones, both with periodontitis. Our results showed the presence of immune complexes in gingiva of nine out of 15 SLE+ patients and in one out of 15 SLE– patients, with significant differences between groups ($p = 0.005$; Fischer Exact test). From the panel of antibodies investigated, IgM was detected in seven SLE+, with weak (+) to strong (+ + +) scores, IgG and IgA were detected in one patient each and C3c was detected in 3 patients, compared to only one patient presenting immunostaining against IgM and C3c in only one SLE– patient (**Supplementary Table 4**).

This finding might aid in the diagnosis of lupus, which is quite difficult to achieve due to the variability and complexity of clinical signs and symptoms, which may also manifest in diseases or conditions others than lupus (1).

The formation of immune complexes is a typical finding in autoimmune diseases. It may arise from circulating antibodies or be formed by the reaction of immunoglobulins with cell or tissue antigens or to bacteria temporarily adsorbed to cells, in a type III hypersensitivity reaction (37). In autoimmune diseases, such as SLE, these reactions typically occur in a granular pattern at the basement membrane (**Figure 3**), as observed in our study. Besides immunoglobulins and complement components, we have also investigated F1b, which was observed in 100% of SLE– and in 93.3% of SLE+ (**Supplementary Table 4**). This marker is associated with revascularization, wound healing and tissue repair and may also be expressed in different disorders, such as chronic kidney disease or amyloidosis.

Deposition of immune complexes in lupus are usually higher at the diagnosis. The presence of lupus-associated autoantibodies may exist in apparently healthy individuals (38), some of which in the pre-clinical phase (1). Antibodies titers and types vary depending on the stage of development of the disease. In the pre-clinical phase, 25% of the population is ANA positive in titers >1:40, 5% in titers ≥1:160 and 2% in titers considered as pathological (39). On the other hand, more than 99% of SLE patients are ANA-positive at some point during the course of the disease, besides the presence of other circulating autoantibodies, such as anti-Ro, anti-La, anti-phospholipids, anti-RNP and anti-dsDNA years before the clinical manifestation of SLE (1). The number of specific antibodies gradually increases in SLE up to the moment of diagnosis, and its accumulation decreases thereafter (40). Three phases are observed in disease development: (1) normal phase in asymptomatic individuals without SLE antibodies; (2) benign immunity, under the influence of genetic and environmental factors, characterized by the presence of autoantibodies (ANA, anti-Ro, anti-La or anti-aPL) in peripheral blood vessels in the absence of clinical manifestations; (3) pathogenic autoimmunity, characterized by

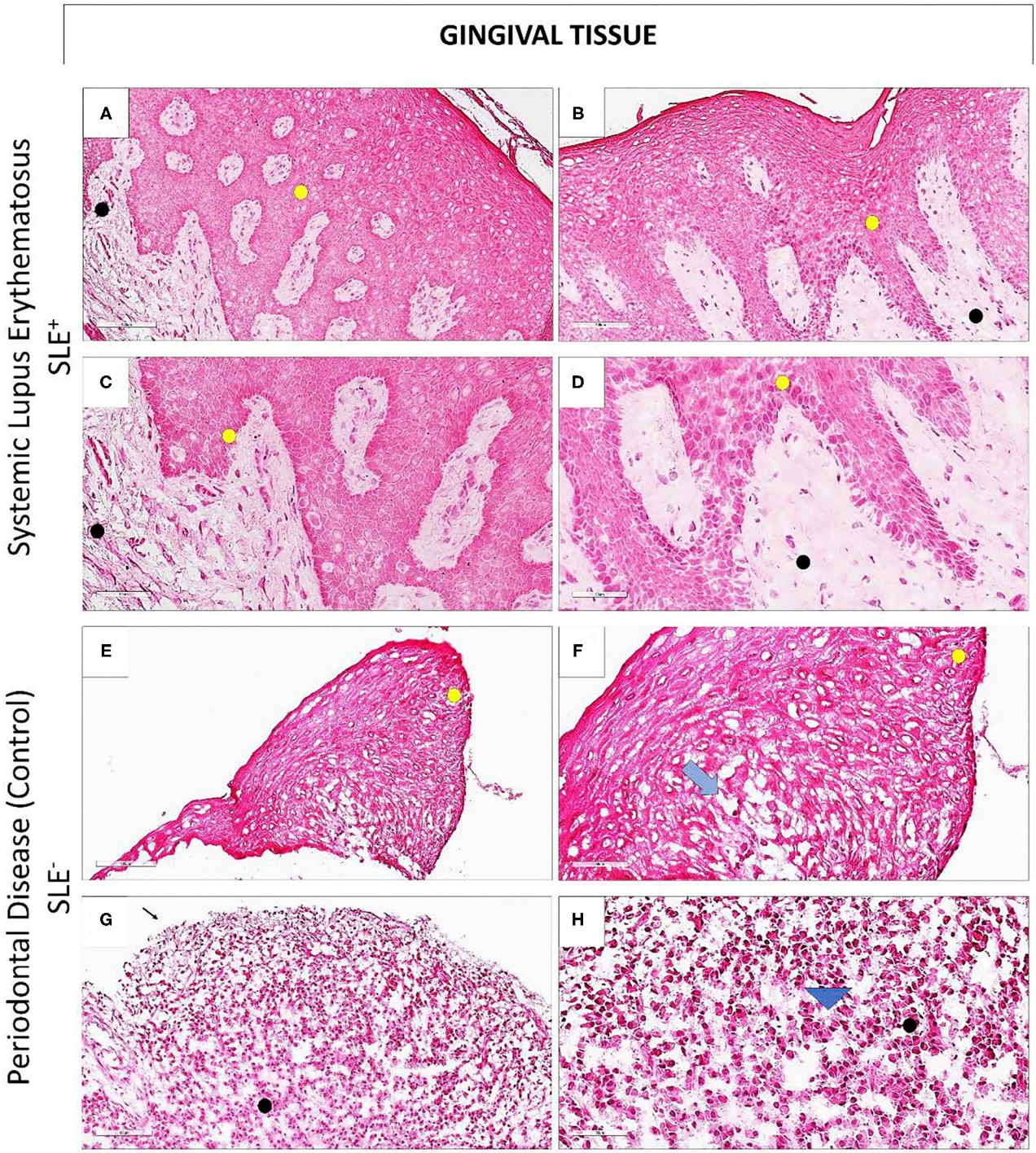

FIGURE 2 | Histopathological findings of gingival biopsies obtained from SLE+ and SLE– women. Microscopic section of the region between the free and attached gingiva of a patient with periodontitis and SLE (**A–D**; SLE+) and a systemically healthy, periodontitis patient (**E–H**; SLE–) **(A,B)**. Note that the gingival mucosa remains healthy, even in the presence of epithelial hiperplasia **(C,D)**; no dysplastic or structural changes in the layers of the epithelium (yellow circle) or in the lamina propria (black circle) were noticed **(E,F)**. In SLE–, periodontitis patient, it is noticed the onset of exocytosis (**F**; blue arrow), and areas of epithelial ulceration **(G)**. A dense inflammatory infiltrate composed mainly by lymphocytes and plasma cells occupying >50% of gingival connective tissue area is noticed (H; arrowhead) (**A,B** and **E,F**: 100μm magnification; **C,D** and **G,H**: 50μm magnification).

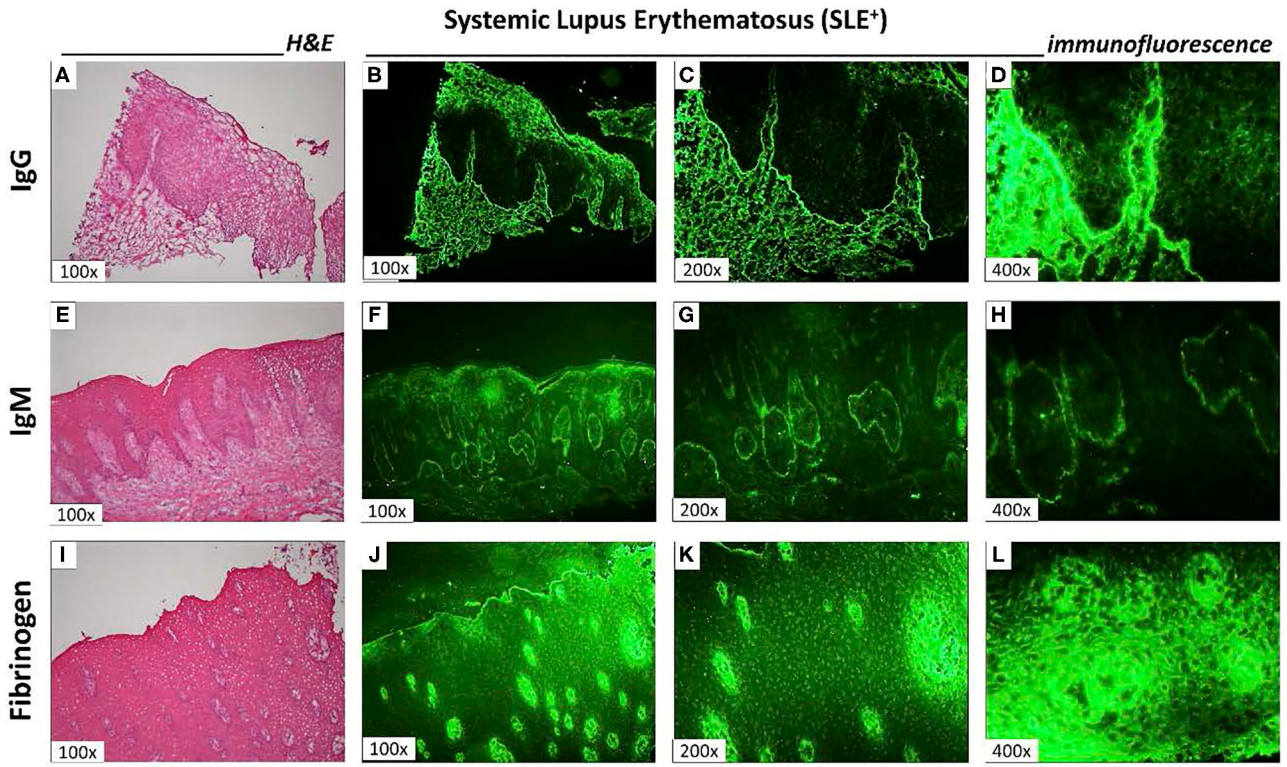

FIGURE 3 | Direct immunofluorescence at LES+ **(A,E,I)**. Hematoxylin and eosin sections obtained from three different SLE+ patients to confirm histopathological diagnosis (100x magnification); Direct immunofluorescence to analyze the presence of IgG (**B** 100x; **C** 200x; **D** 400x magnification of gingival biopsy shown in **A**); IgM (**F** 100x; **G** 200 x; **H** 400 x magnification of gingival biopsy shown in **E**) and fibrinogen (**J** 100x; **K** 200x; **L** 400x magnification of section shown in **I**). The immunoglobulin immunostaining was granular in appearance, focusing mainly on areas of loose connective tissue near the basement membrane. However, the detection of fibrinogen has also been found in epithelial cells **(L)**.

the presence of anti-dsDNA, anti-Sm and anti-RNP and the development of clinical signs and symptoms (1).

Our study included women with established diagnosis and in treatment of SLE. Therefore, in these patients, deposition of autoantibodies in organs and tissues had already taken place. Even so, the presence of higher levels of immunoglobulins and complement in the basement membrane of SLE+ periodontitis patients, especially IgM, provides evidence of the disease, and might be helpful for the diagnosis of SLE or its flare. Higher titers of circulating antibodies, low C3, C4 and CH50 and increased deposition of immune complexes in tissues are seen during lupus activity, especially on those who develop LN, tending to normalize with clinical improvement (9, 41, 42).

Patients with active kidney disease tended to have lower levels of CH50 and C3 and higher levels of immune complexes detected by C1qBA than those with extra-renal manifestations only. Patients with renal and extrarenal manifestations have lower levels of CH50, C4 and C3, but the deposition of immune complexes in such cases is lower than those observed in LN. These findings highlight the concept that SLE and LN are two autoimmune conditions characterized by isotype specificity of auto-antibodies (42). In our sample, 5 patients had renal or extra-renal diseases and, from that, 2 presented IgM immunostaining and 1 C3. No patient showed positive staining for C1q in neither groups (**Supplementary Table 4**). Low levels of complement without high levels of C1q suggest unlikely kidney disease (9). Additionally, it could be observed that SLE-A patients showed significantly higher titers of anti-dsDNA and lower C4 levels than SLE-I (**Table 2**), corroborating these findings.

Different studies showed increased serum IgG titers against periodontal pathogens in chronic periodontitis (16, 18–20) and

TABLE 5 | Quantitative analysis of direct immunofluorescence.

	SLE+ [median; (mean ± sd)]	SLE– [median; (mean ± sd)]	*p*-value
IgG	0 (0.13 ± 0.51)	0 (0)	>0.99
IgM	0 (0.60 ± 0.82)	0 (0.06 ± 0.25)	**0.03**
IgA	0 (0.06 ± 0.25)	0 (0)	>0.99
C1q	0 (0)	0 (0)	>0.99
C3	0 (0.20 ± 0.41)	0 (0.06 ± 0.25)	0.59
F1b	2 (1.46 ± 0.63)	1 (1.20 ± 0.41)	0.12
Total	2 (2.46 ± 1.55)	1 (1.33 ± 0.81)	**0.01**

**Mann Whitney; significant if p < 0.05. Bold values - significant differences between groups.*

even higher titers in gingival crevicular fluid (43). Immune complexes and IgG deposits with active complement factors were noticed in periodontitis patients (15, 17), associated with increased number of osteoclasts in alveolar bone crest, suggesting their involvement in the acute phase of periodontal destruction (21).

Histopathological findings of gingival biopsies obtained from SLE+ patients showed minimal amount of inflammatory infiltrate at gingival connective tissue in periodontitis patients (**Figure 2**), contrasting with the findings of SLE– periodontitis patients, who showed a dense infiltrate predominantly composed of lymphocytes and plasma cells (44). This can be explained by the use of immunosuppressants or immunomodulators for lupus control. Only one SLE-I did not use any of these drugs (**Supplementary Table 3**). A positive association between low daily doses of prednisone (<10 mg) and CAL $\leq$ 3 mm was observed (**Table 4**). This finding emphasizes the role of immune inflammatory host responses in the pathogenesis of periodontitis.

The suppression or modulation of immune inflammatory host response correlates with clinical periodontal parameters. Worse periodontal conditions, especially PD, CAL, FMBS and percentage of sites with PD $\geq$5 mm, were observed in SLE– than in SLE+ women (**Table 3**), in spite of similar overall characteristics of SLE+ and SLE– (**Table 1**). These findings differ from other studies which showed worse periodontal conditions in SLE patients compared to healthy ones (31, 45, 46). Gofur et al. (47) showed that worst periodontal conditions were associated with higher SLEDAI scores. However, the maximum SLEDAI in our sample was 28 (data not shown), which may account for differences observed between our and other studies. Other reports, however, did not find differences in periodontal parameters of SLE patients (27, 45, 48).

No differences in the prevalence of healthy/periodontitis patients were observed in SLE+ and SLE- (**Supplementary Table 2**). In SLE+, 2 patients (8%) and 1 (4%) were classified as periodontally healthy or with gingivitis, respectively, according to 2018 AAP/EFP classification of periodontal and peri-implant diseases and conditions (36). In SLE–, five patients (20%) were classified as healthy, with no significant differences between groups. A recent systematic review and meta-analysis (49) including eight case-control studies with 487 cases of SLE and 1383 participants in total, found that the risk of periodontitis in cases of SLE was significantly higher than in systemically healthy controls, with RR of 1.76 (95% CI 1.29–2.41; $p = 0.0004$). However, no significant differences were observed between groups in relation to periodontal measures, such as probing depth and loss of clinical insertion, as also observed in our study.

Pessoa et al. (25) evaluated the reciprocal impact of the subgingival microbiota on systemic inflammation in patients with SLE. Ninety-one women were recruited, 31 of whom were systemically healthy, 29 with inactive SLE and 31 with active SLE. There was a high expression of pro-inflammatory cytokines in patients with SLE compared to healthy controls. In SLE-I, low-intensity inflammation was observed, while a potent anti-inflammatory cytokine, IL-10, attenuated clinical phenotypes. Of 24 significant oral microbial abundances found in patients with SLE, 14 unique subgingival bacterial profiles were elevated in SLE, with a particular increase in the levels of *T. denticola* and *T. forsythia* in patients with SLE-A compared to control. The cytokine-bacteria correlations The correlation between cytokines and bacteria showed that periodontal pathogens dominating the environment increased systemic levels of cytokines. Deeper bags and greater loss of insertion were observed in SLE patients, especially SLE-I, possibly due to chronic, long-lasting, low-intensity inflammation. Thus, taking into account the results of another study (45), it can be hypothesized that, although there is dysbiosis in patients with SLE, the clinical manifestation of periodontitis in these patients is masked by the use of corticosteroids and immunosuppressants.

As far as we know, no study has investigated lupus-related deposition of antibodies in gingival tissues. Additionally, to date, no serum or urine biomarker is sufficiently accurate in the diagnosis of incipient or recurrent LN so that renal biopsies could be replaced (50, 51). Our findings, although interesting, should be further investigated, since all patients were diagnosed and in long-term treatment for active or inactive lupus, which might contribute not only to decreased deposition of immune complexes at gingival tissues as well as to better clinical parameters in test group. Considering so, further studies are necessary to confirm or discard our hypothesis that lupus-associated immune complexes deposit in gingival tissues and may aid in the diagnosis of the disease or its flare.

The findings of this study suggest that lupus-associated immune complexes can be detected by direct immunofluorescence in biopsies of gingiva, which could aid in diagnosis of the disease. Besides that, the use of immunosuppressants and immunomodulators limits density and extension of the inflammatory infiltrate at gingival tissues, contributing to a better clinical periodontal condition.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by School of Dentistry at Bauru – USP Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS, LP, and SG conceived and designed the experiments. JP performed the experiments and tabulated data. MN, AN, and DD performed histopathologic and direct immunofluorescence assays. MZ, CD, and MR contributed in control group treatment.

AS, JP, and LP wrote the paper. 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.2021.591236/full#supplementary-material

REFERENCES

1. Ugarte-Gil MF, González LA, Alarcón GS. Lupus: the new epidemic. *Lupus*. (2019) 28:1031–50. doi: 10.1177/0961203319860907
2. Wallace D, Hahn BH. *Dubois'Lupus Erythematosus and Related Syndromes. Elsevier Health Sciences*. Amsterdam: Elsevier (2012).
3. Lim SS, Bayakly AR, Helmick CG, Gordon C, Easley KA, Drenkard C. The incidence and prevalence of systemic lupus erythematosus, 2002–2004: the Georgia Lupus Registry. *Arth Rheumatol*. (2014) 66:357–68. doi: 10.1002/art.38239
4. Vilar MJP, Sato EI. Estimating the incidence of systemic lupus erythematosus in a tropical region (Natal, Brazil). *Lupus*. (2002) 11:528–32. doi: 10.1191/0961203302lu244xx
5. Roper G. *Lupus Awareness Survey for the Lupus Foundation of America*. Washington, DC: GfK Roper Public Affairs and Corporate Communications (2012).
6. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum*. (1982) 25:1271–7. doi: 10.1002/art.1780251101
7. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum*. (1997) 40:1725. doi: 10.1002/art.1780400928
8. Petri M, Orbai A-M, Alarcón GS, Gordon C, Merrill JT, Fortin PR, et al. Derivation and validation of the systemic lupus international collaborating clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum*. (2012) 64:2677–86. doi: 10.1002/art.34473
9. Lloyd W, Schur PH. Immune complexes, complement and anti-DNA in exacerbations of systemic lupus erythematosus (SLE). *Medicine*. (1981) 60:208–17. doi: 10.1097/00005792-198105000-00004
10. Aringer M, Costenbader K, Brinks R, Boumpas D, Daikh H, Jaine D, et al. OP0020 Validation of new systemic lupus erythematosus classification criteria. *Ann Rheum Dis*. (2018) 77:60. doi: 10.1136/annrheumdis-2018-eular.3679
11. Yung S, Chan TM. Anti-dsDNA antibodies and resident renal cells – their putative roles in pathogenesis of renal lesions in lupus nephritis. *Clin Immunol*. (2016) 185:40–50. doi: 10.1016/j.clim.2016.09.002
12. Cameron JS. Lupus nephritis. *J Am Soc Nephrol*. (1999) 10:413–24.
13. Nozaki Y. The network of inflammatory mechanisms in lupus nephritis. *Front Med*. (2020) 7:591724. doi: 10.3389/fmed.2020.591724
14. Almaani S, Meara A, Rovin BH. Update in lupus nephritis. *Clin J Am Soc Nephrol*. (2017) 12:825–35. doi: 10.2215/CJN.05780616
15. Toto PD, Lin LM, Gargiulo AW. Immunoglobulins and complement in human periodontitis. *J Periodontol*. (1978) 49:631–4. doi: 10.1902/jop.1978.49.12.631
16. Mouton C, Hammond PG, Slots J, Genco RJ. Serum antibodies to oral bacteroides asaccharolyticus (Bacteroides gingivalis): relationship to age and periondontal disease. *Infect. Immun*. (1981) 31:182–92. doi: 10.1128/IAI.31.1.182-192.1981
17. Nikolopoulou-Papaconstantinou AA, Johannessen AC, Kristoffersen T. Deposits of immunoglobulins, complement, and immune complexes in inflamed human gingiva. *Acta Odontol Scand*. (1987) 45:187–93. doi: 10.3109/00016358709098858
18. Ebersole JL. Systemic humoral immune responses in periodontal disease. *Crit Rev Oral Biol Med*. (1990) 1:283–331. doi: 10.1177/10454411900010040601
19. Taubman MA, Haffajee AD, Socransky SS, Smith DJ, Ebersole JL. Longitudinal monitoring of humoral antibody in subjects with destructive periodontal diseases. *J Periodontal Res*. (1992) 27:511–21. doi: 10.1111/j.1600-0765.1992.tb01825.x
20. McArthur WP, Clark WB. Specific antibodies and their potential role in periodontal diseases. *J Periodontol*. (1993) 64:807–18. doi: 10.1902/jop.1993.64.8s.807
21. Kuramoto A, Yoshinaga Y, Kaneko T, Ukai T, Shiraishi C, Oshino K, et al. The formation of immune complexes is involved in the acute phase of periodontal destruction in rats. *J Periodontal Res*. (2012) 47:455–62. doi: 10.1111/j.1600-0765.2011.01453.x
22. Gonzales TS, Coleman GC. Periodontal manifestations of collagen vascular disorders. *Periodontology 2000*. (1999) 21:94–105. doi: 10.1111/j.1600-0757.1999.tb00170.x
23. Marques CPC, Maor Y, De Andrade MS, Rodrigues VP, Benatti BB. Possible evidence of systemic lupus erythematosus and periodontal disease association mediated by toll-like receptors 2 and 4. *Clin Exp Immunol*. (2016) 183:187–92. doi: 10.1111/cei.12708
24. Sete MRC, Figueredo CMS, Sztajnbok F. Periodontal disease and systemic lupus erythematosus. *Rev Bras Reumatol*. (2015) 56:165–70. doi: 10.1016/j.rbre.2015.09.001
25. Pessoa L, Aleti G, Choudhury S, Nguyen D, Yaskell T, Zhang Y, et al. Host-microbial interactions in systemic lupus erythematosus and periodontitis. *Front Immunol*. (2019) 10:2602. doi: 10.3389/fimmu.2019.02602
26. Mutlu S, Richards A, Maddison P, Scully C. Gingival and periodontal health in systemic lupus erythematosus. *Comm Dental Oral Epidemiol*. (1993) 21:158–61. doi: 10.1111/j.1600-0528.1993.tb00742.x
27. Pessoa LCM. *Clinical and Microbiologic Analysis of Periodontal Disease in Patients With Systemic Lupus Erythematosus*. Bauru: Faculty of Dentistry at Bauru, University of São Paulo (2016). 95 p. (In portuguese).
28. Kobayashi T, Ito S, Yasuda K, Kuroda T, Yamamoto K, Sugita N, et al. The combined genotypes of stimulatory and inhibitory Fcy receptors associated with systemic lupus erythematosus and periodontitis in Japanese adults. *J Periodontol*. (2007) 78:467–74. doi: 10.1902/jop.2007.060194
29. Novo E, Garcia-MacGregor E, Viera N, Chaparro N, Crozzoli Y. Periodontitis and anti-neutrophil cytoplasmic antibodies in systemic lupus erythematosus and rheumatoid arthritis: a comparative study. *J Periodontol*. (1999) 70:185–8. doi: 10.1902/jop.1999.70.2.185
30. Fabbri C, Fuller R, Bonfá E, Guedes LK, D'alleva PS, Borba EF. Periodontitis treatment improves systemic lupus erythematosus response to immunosuppressive therapy. *Clin Rheum*. (2014) 33:505–9. doi: 10.1007/s10067-013-2473-2
31. Wang CY, Chyuan I-T, Wang Y-L, Kuo M-YP, Chang CW, Wu K-J, et al. β2-glycoprotein I-dependent anti-cardiolipin antibodies associated with periodontitis in patients with systemic lupus erythematosus. *J Periodontol*. (2015) 86:995–1004. doi: 10.1902/jop.2015.140664

32. Calderaro DC, Ferreira GA, Corrêa JD, Mendonça SMS, Silva TA, Costa FO, et al. Is chronic periodontitits premature in systemic lupus erythematosus patients? *Clin Rheum*. (2016) 36:713–8. doi: 10.1007/s10067-016-3385-8
33. Gladman D, Ginzler E, Goldsmith C, Fortin P, Liang M, Sanchez-Guerrero J, et al. The development and initial validation of the systemic lupus international collaborating clinics/American college of rheumatology damage index for systemic lupus erythematosus. *Arthritis Rheum.* (1996) 39:363–9. doi: 10.1002/art.1780390303
34. Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH, Austin A, et al. Derivation of the sledai A disease activity index for lupus patients. *Arthritis Rheum*. (1992) 35:630–40. doi: 10.1002/art.1780350606
35. Petri M, Purvey S, Fang H, Magder LS. Predictors of organ damage in systemic lupus erythematosus. *Arth Rheum*. (2012) 64:4021–8. doi: 10.1002/art.34672
36. Tonetti MS, Greenwell H, Kornman KS. Staging and grading of periodontitis: framework and proposal of a new classification and case definition. *J Periodontol*. (2018) 89:S159–72. doi: 10.1002/JPER.18-0006
37. Albilia JB, Lam DK, Clockie CLM, Sandór GKB. Systemic lupus erythematosus: a review for dentists. *J Can Dent Assoc.* (2007) 73:823–8.
38. Lu R, Munroe ME, Guthridge JM, Bean KM, Fife DA, Chen H, et al. Dysregulation of innate and adaptive serum mediators precedes systemic lupus erythematosus classification and improves prognostic accuracy of autoantibodies. *J Autoimm*. (2016) 74:182–93. doi: 10.1016/j.jaut.2016.06.001
39. Olsen NJ, Karp DR. Autoantibodies and SLE: the threshold for disease. *Nat Rev Rheum*. (2014) 10:181–6. doi: 10.1038/nrrheum.2013.184
40. Arbuckle MR, Mcclain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med*. (2003) 349:1526–33. doi: 10.1056/NEJMoa021933
41. Barnett E, Knutson D, Abrass C, Chia D, Young L, Liebling M. Circulating immune complexes: their immunochemistry, detection and importance. *Ann Int Med*. (1979) 91:430–40. doi: 10.7326/0003-4819-91-3-430
42. Bruschi M, Petretto A, Vaglio A, Santucci L, Candiano G, Ghiggeri GM. Annexin A1 and autoimmunity: from basic science to clinical applications. *Int J Mol Sci*. (2018) 19:1348. doi: 10.3390/ijms19051348
43. Tew JG, Marshall DR, Burmeister JA, Ranney RR. Relationship between gingival crevicular fluid and serum antibody titers in young adults with generalized and localized periodontitis. *Infect Immun*. (1985) 49:487–93. doi: 10.1128/IAI.49.3.487-493.1985
44. Page RC, Schroeder HE. Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest*. (1976) 34:235–49.
45. Corrêa JD, Calderaro DC, Ferreira GA, Mendonça SMS, Fernandes GR, Xiao E, et al. Subgingival microbiota dysbiosis in systemic lupus erythematosus: association with periodontal status. *Microbiome*. (2017) 5:34. doi: 10.1186/s40168-017-0252-z
46. Fernandes EGC, Savioli C, Siqueira JTT, Silva CAA. Oral health and the masticatory system in juvenile systemic lupus erythematosus. *Lupus*. (2007) 16:713–9. doi: 10.1177/0961203307081124
47. Gofur NRP, Handono K, Kalim H. Oral manifestation on systemic lupus erythematosus patients. *J Publ Health Clin Sci*. (2019) 6:172–8. doi: 10.32827/ijphcs.6.3.172
48. Al-Mutairi KD, Al-Zahrani MS, Bahlas SM, Kayal RA, Zawawi KH. Periodontal findings in systemic lupus erythematosus patients and healthy controls. *Saudi Med J*. (2015) 36:463–8. doi: 10.15537/smj.2015.4.10746
49. Rutter-Locher Z, Smith TO, Giles I, Sofat N. Association between systemic lupus erythematosus and periodontitis: a systematic review and meta-analysis. *Front Immunol*. (2017) 8:1295. doi: 10.3389/fimmu.2017.01295
50. Bakshi J, Segura BT, Wincup C, Rahman A. Unmet needs in the pathogenesis and treatment of systemic lupus erythematosus. *Clin Rev Allerg Immunol.* (2018) 55:352–67. doi: 10.1007/s12016-017-8640-5
51. Davis P, Cumming RH, Verrier-Jones J. Relationship between anti-DNA antibodies complement consumption and circulating immune complexes in systemic lupus erythematosus. *Clin Exp Immunol*. (1977) 28:226–32.

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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Vaccination With the Commensal *Streptococcus mitis* Expressing Pneumococcal Serotype 5 Capsule Elicits IgG/IgA and Th17 Responses Against *Streptococcus pneumoniae*

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Recent studies have identified a clinical isolate of the commensal *Streptococcus mitis* that expresses *Streptococcus pneumoniae* serotype 5 capsule (*S. mitis* serotype 5) and shows serospecificity toward pneumococcal serotype 5. However, it remains unknown whether *S. mitis* serotype 5 induces protective immunity against pneumococcal serotype 5. In this study, we evaluated the ability of *S. mitis* serotype 5 to generate protective immunity in a mouse model of lung infection with pneumococcal serotype 5. Upon challenge infection with *S. pneumoniae* serotype 5, mice intranasally immunized with *S. mitis* serotype 5 exhibited reduced pneumococcal loads in the lungs, nasal wash, and bronchoalveolar lavage fluid compared with those receiving PBS (control). The immunized mice displayed significantly higher levels of IgG and IgA antibodies reactive to *S. mitis* serotype 5, *S. pneumoniae* serotype 5 or *S. pneumoniae* serotype 4 than the antibody levels in control mice. In vaccinated mice, the IgG/IgA antibody levels reactive to *S. mitis* serotype 5 or S. *pneumoniae* serotype 5 were higher than the levels reactive to *S. pneumoniae* serotype 4. Furthermore, *in-vitro* restimulation of the lung-draining mediastinal lymph node cells and splenocytes from immunized mice with killed *S. mitis* serotype 5, *S. pneumoniae* serotype 5 or *S. pneumoniae* serotype 4 showed enhanced Th17, but not Th1 and Th2, responses. Overall, our findings show that mucosal immunization with *S. mitis* serotype 5 protects against *S. pneumoniae* serotype 5 infection and induces Th17 and predominant serotype-specific IgG/IgA antibody responses against pneumococcal infection.

Keywords: *Streptococcus mitis*, Immunity, Antibody, Th17, *Streptococcus pneumoniae*

INTRODUCTION

Streptococcus pneumoniae is an important human pathogen that causes a range of diseases, including sepsis, meningitis, and pneumonia, and poses a threat to public health worldwide (1, 2). According to the World Health Organization, *S. pneumoniae* is responsible for approximately 1.6 million deaths per annum, particularly among young children (1, 3). *S. pneumoniae*'s capsular

polysaccharides are considered to be the most important virulence factor by protecting pneumococci from immune cell-mediated phagocytosis. A pneumococcal serotype is characterized by serological reactivity to and molecular structure of its capsular polysaccharides. The immunity ensued against the capsular serotype is protective and has crucial implications for vaccine development (4–6). More than 90 serotypes have thus far been reported (4). Out of these pneumococcal serotypes, serotype 5 is strongly associated with invasive pneumococcal disease (IPD), with an invasiveness index that is 60 times higher than those of some of the least invasive serotypes (7, 8). And so, this serotype is contained in pneumococcal conjugate vaccine (PCV) and pneumococcal polysaccharide vaccine (PPSV) formulations.

Streptococcus mitis, a commensal bacterium, colonizes the mucosal surfaces of the human oral cavity and upper respiratory tract, and shares a major proportion of its genome with *S. pneumoniae* (9, 10). Our previous studies using humans and animal models have reported that antibody (IgG/IgA) and T helper cell (Th) 17 (Th17) responses specific for *S. mitis* show cross-reactivity with pneumococcal serotypes (11–13). Intranasal immunization of mice with live *S. mitis* triggered serotype-independent immunity against pneumococcal lung infection (13). Upon vaccination with genetically engineered *S. mitis* that expresses pneumococcal serotype 4 capsule (*S. mitis* TIGR4cps), mice generated enhanced protection against *S. pneumoniae* serotype 4 in a serotype-dependent fashion (13). Recently, Pimenta *et al.* have recovered commensal isolates of mitis streptococci from the upper respiratory tract of adult individuals that were PCR-positive for the pneumococcal serotype 5 specific gene (*wzy*5) (14). The *S. mitis* (hereinafter called as *S. mitis* serotype 5) shared the highest similar capsular polysaccharide biosynthetic gene cluster (*cps*5) with the same order in pneumococcal serotype 5 (14). Furthermore, antisera specific for *S. mitis* serotype 5 showed reactivity with pneumococcal serotype 5, indicating a positive Quellung reaction, and induced serotype 5-specific opsonophagocytosis (14).

In the present study, we specifically aimed to investigate whether *S. mitis* serotype 5 confers protective immunity against *S. pneumoniae* using a mouse model of pneumococcal lung infection. We also assessed the associated adaptive immune (IgG/IgA and Th) responses ensued due to immunization with *S. mitis* serotype 5. Our findings from this study provide important insights into how the naturally occurring commensal *S. mitis* that expresses pneumococcal serotype 5 can be used to generate protective immunity against infections with *S. pneumoniae*, which may have implications for the development of vaccines that contain serotypes that are prevalent in a particular geographical area.

MATERIALS AND METHODS

Bacterial Strains and Media

S. mitis serotype 5 (KE67013) was kindly provided by the CDC, Atlanta, USA (14). *S. pneumoniae* serotypes included were *S. pneumoniae* serotype 5 (ATCC 6305; CCUG 33774) and *S. pneumoniae* serotype 4 (TIGR4). The bacterial strains were suspended in trypticase soy broth (Becton Dickinson, Franklin Lakes, NJ, USA) and 15% glycerol and stored in −80°C freezer. For the use of bacteria, stock cultures were diluted and grown at 37°C to an optical density (OD) of 0.5 at 600 nm in a 5% CO_2 incubator. The bacterial cells were harvested by centrifugation at 5,000 *g* for 10 min at 4°C and washed in endotoxin free Dulbecco's-PBS (Sigma-Aldrich, St. Louis, MO, USA).

Mice

Swiss mice used in this study were females of 6-8 weeks age. These mice were specific pathogen free (SPF) and bought from the JANVIER LABS, France, and quarantined and housed in a Minimal Disease Unit at the animal facility at Oslo University Hospital, Rikshospitalet, Oslo, Norway. The mice were kept in isocages that are environmentally enriched with impellers and paper nest building, and given standard feed and water ad libitum. All mouse experiments were approved by the Norwegian Food Safety Authority, Oslo, Norway (Project license number FOTS – 22302) and performed in accordance with the guidelines of the Norwegian Animal Welfare Act (10 June 2009 no. 97), the Norwegian Regulation on Animal Experimentation (REG 2015-06-18-761) and the European Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes. Mice were allowed a one week acclimatization period before experiments were started.

Immunization and Challenge Infection

To perform immunization, mice were anesthetized with isoflurane (4%), followed by intranasal administration of 5 x 10^7 colony forming units (CFU) of *S. mitis serotype 5* in 20 µl of PBS or 20 µl of PBS (control) for each mouse at days 0, 14, and 21. The immunized mice were anesthetized with isoflurane (4%) at 24 hours after the last immunization, followed by intranasal instillation with 8 x 10^6 CFU of *S. pneumoniae* serotype 5 suspended in 50 µl of PBS, as described previously (13). Of note, we performed our experiment with 4 mice in immunized and 4 mice in control group, and the experiment was repeated to confirm the findings. The data represented in figures are pooled from the results of these two independent experiments.

Sample Collection

Mice were euthanized at 24 hours after pneumococcal challenge, and the nasal wash, bronchoalveolar lavage fluid (BALF), spleen, blood, lungs, and lung-draining mediastinal lymph nodes were collected and stored in ice for further processing. For euthanasia, mice were anesthetized with isoflurane (4%) and then inoculated with an intraperitoneal injection of pentobarbital (0.5 ml per mouse). To obtain antisera, the freshly isolated blood was kept at 4°C for 1 hour and then centrifuged at 1000g for 5 minutes. The supernatant antisera were collected and preserved at -80°C freezer for analysis. The nasal wash, BALF, and lungs were collected from the euthanized mice, as described previously (15). To recover the BALF and nasal wash, a small cut in the trachea was made with a scissor and 1 ml of sterile cold PBS was

inoculated with a syringe (19 gauge needle) and recovered for plating as well as antibody measurements. The lungs were mashed on a 70 μm cell strainer (ThermoFisher Scientific, Rockford, IL, USA) with the plunger of a 3 ml syringe and washed with 1 ml PBS for CFU counting and cytokine analysis. The nasal wash, BALF, and lung samples were plated onto blood agar plates containing gentamicin (5 $\mu g \cdot ml^{-1}$) for differentiation from other species and CFU calculation (13, 15).

Measurement of Antibody Responses

To determine antibody levels in mouse samples, a whole cell ELISA was used as described previously (12, 13). In brief, each well of a 96-well plate (Maxisorb, Nunc, Thermo Scientific) was coated overnight with 100 μl of bacterial suspension (OD_{600} 0.5), which was washed and then fixed with 10% formalin. The plate was washed and blocked with a blocking buffer (PBS + 0.05% Tween + 1% BSA) and incubation for 1 h at 37°C. Sera (1:1000), BALF (1:10) and nasal wash (1:10) were diluted, and added to wells in duplicate, incubated for 2 hours at room temperature before addition of the anti-IgG/HRP or anti-IgA/HRP secondary antibody (1:10000) followed by incubation for 2 h at room temperature. The plates were washed and 100 μl of TMB substrate (ThermoFisher Scientific, Rockford, IL, USA) was added to each well. The plates were incubated in the dark at room temperature for 15 min, after which stop solution (ThermoFisher Scientific, Rockford, IL, USA) was added to each well to terminate the reaction. Absorbance was measured by reading the plates at 450 nm using a Multi-Mode reader (BioTek™ Cytation™ 3; ThermoFischer Scientific).

Cell Isolation From the Lymph Nodes and Spleen

The mediastinal lymph nodes and spleen were removed from the euthanized mice and processed into single-cell suspensions. The mediastinal lymph nodes and spleen were mashed on a 70 μm cell strainer (ThermoFisher Scientific, Rockford, IL, USA) with the plunger of a 3 ml syringe and washed with the washing buffer (PBS, 0.5% BSA and 5 mM EDTA). The cell suspension was lysed with RBC lysis buffer and washed twice. Hemocytometer and trypan blue were used to count viable cells.

In Vitro Restimulation of the Lymph Node Cells and Splenocytes

5 x 10^6 splenocytes in one ml or 1 x 10^6 lymph node cells in 200μl of complete RPMI medium (10% heat-inactivated FBS, 25 μg/mL gentamicin, L-glutamine, and sodium bicarbonate; Sigma-Aldrich, UK) were cultured at 37°C, and restimulated with UV-killed *S. mitis* serotype 5, *S. pneumoniae* serotype 5 or *S. pneumoniae* serotype 4 (10^5 CFU/ml) for 72 hours. The culture supernatants were frozen at -80^0C for further cytokine analysis by commercial ELISA kits. The concentrations of IFN-γ, IL-4, and IL-17A were measured by Ready-SET-Go ELISA kits (eBioscience, San Diego, CA, USA), whereas the concentration of IL-4 was measured with an ELISA kit from Invitrogen (Vienna, Austria), in accordance with the instructions of respective manufacturers. The cytokine detection limit of the ELISA kits for IL-17, IL-4, and IFN-γ were 4, 3.9, and 15 pg/ml, respectively. Similarly, the cytokine levels in the lung homogenates, nasal wash and BALF were analyzed.

Statistics

Unpaired Student's *t* test was used for comparing two groups (GraphPad Prism Software, version 7, Graph Pad, San Diego, CA, USA). A *p* value less than 0.05 was considered significant.

RESULTS

Intranasal Immunization of Mice With *S. mitis* Serotype 5 Protects Against Challenge Infection With *S. pneumoniae* Serotype 5

Accumulating evidence has shown that *S. mitis* serotype 5 exhibits antigenic relatedness to pneumococcal serotype 5 (14). To assess the protective efficacy of *S. mitis* serotype 5, we intranasally immunized mice with live *S. mitis* serotype 5 thrice followed by lung infection with *S. pneumoniae* serotype 5. The immunized mice exhibited significantly reduced pneumococcal loads in the nasal wash, BALF, and lungs compared to the control mice receiving PBS (**Figure 1**). Of note, *S. mitis* serotype 5 immunization completely eliminated pneumococcal burden from the BALF of 50% of all the mice immunized, whereas none of the control mice was able to totally clear pneumococcal loads from any of the samples collected. Thus, *S. mitis* serotype 5 confers strong protection against lung infection with *S. pneumoniae* serotype 5. Of note, a table showing CFU counts from each mouse used in this study has been included (**Supplementary Table 1**).

S. mitis Serotype 5 Induces Predominant Serotype-Specific IgG and IgA Antibody Responses

Our recent findings demonstrate that IgG and IgA antibodies from mice immunized with the *S. mitis* type strain, which has a capsule locus not found in *S. pneumoniae*, reveal reactivity with *S. pneumoniae* serotypes 2 and 4, suggesting an antibody-mediated response that is independent of the pneumococcal serotype (13). It is further shown that antisera raised against *S. mitis* serotype 5 show serotype-specific reactivity with *S. pneumoniae* serotype 5 (14). To have detailed knowledge on how IgG and IgA antibodies specific for *S. mitis* serotype 5 react with different pneumococcal serotypes, we immunized mice with *S. mitis* serotype 5, which was followed by lung infection with *S. pneumoniae* serotype 5, and examined IgG and IgA responses in the nasal wash, BALF, and sera using a whole cell ELISA. The mice immunized with *S. mitis* serotype 5 displayed significantly higher levels of IgG antibodies binding to *S. mitis* serotype 5, *S. pneumoniae* 4 or *S. pneumoniae* 5 compared with IgG levels in control mice (**Figure 2A**). However, IgG levels in immunized mice reactive to *S. pneumoniae* 4 were much lower than the IgG levels binding to *S. mitis* serotype 5 or *S. pneumoniae* 5 (**Figure 2A**). In accordance with these findings, the immunized mice exhibited increased binding of IgA antibodies to

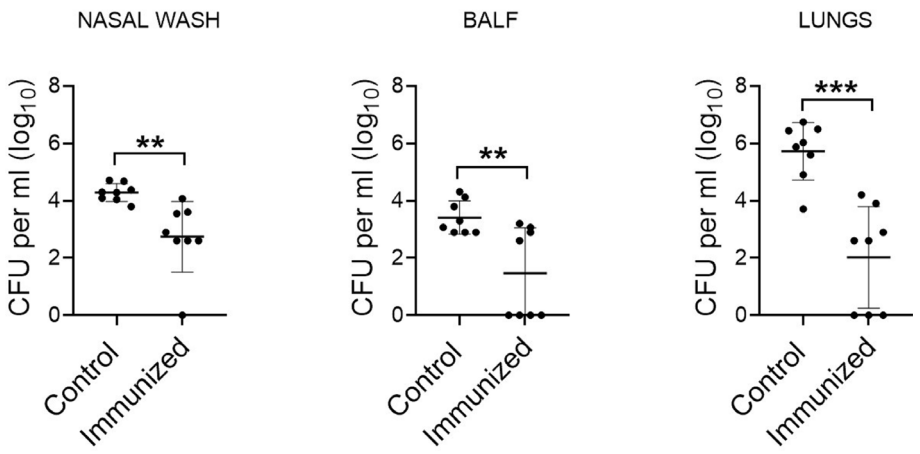

FIGURE 1 | Protective efficacy of *S. mitis* serotype 5 against lung infection with pneumococcal serotype 5. Mice were intranasally immunized with *S. mitis* serotype 5 or PBS (control) at days 0, 14, and 21, and then subjected to lung infection with *S. pneumoniae* serotype 5. They were sacrificed at 24 hours following the challenge infection, and nasal wash, BALF, and lungs were collected for the analysis of pneumococcal load (CFU). Data are shown as mean ± SD and pooled from the results of two independent experiments with 4 mice in each group. Each symbol represents data from an individual mouse, and the horizontal bars are mean values of the groups. **p<0.01; ***p<0.001. Unpaired Student's *t* test.

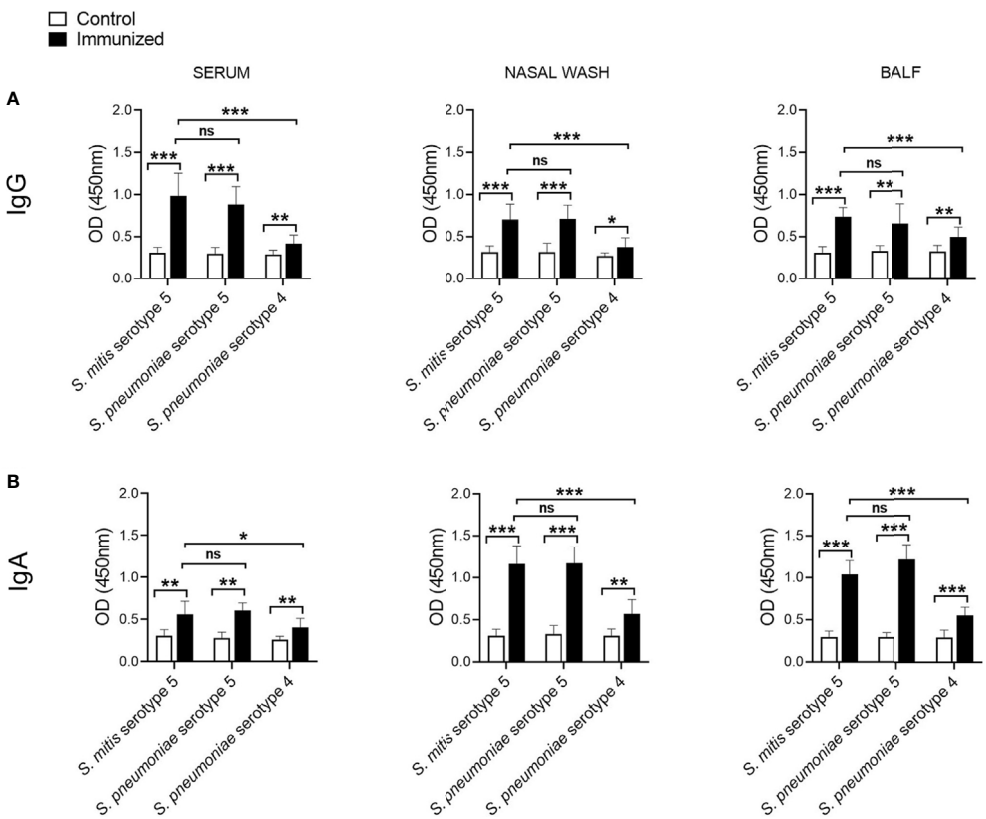

FIGURE 2 | IgG and IgA responses to *S. pneumoniae* following immunization with *S. mitis* serotype 5. Mice were immunized with *S. mitis*, which was followed by pneumococcal lung infection, and the nasal wash, BALF and sera samples were collected to analyze **(A)** IgG and **(B)** IgA responses. Levels of IgG and IgA antibodies reactive to pneumococcal serotypes in the sera were measured by whole cell ELISA. The sera were diluted 1:1000, and the nasal wash and BALF 1:10. Data are shown as mean ± SD and pooled from the results of two independent experiments with 4 mice in each group. *p<0.05; **p<0.01; ***p<0.001. Unpaired student's *t* test. ns, not significant.

S. pneumoniae 5 or *S. pneumoniae* 4 compared with control mice (**Figure 2B**). And, IgA levels binding to *S. mitis* serotype 5 or *S. pneumoniae* 5 were significantly higher than the levels reactive to *S. pneumoniae* 4 (**Figure 2B**). Cumulatively, intranasal immunization with *S. mitis* serotype 5 induces mucosal and systemic IgG and IgA antibody responses to pneumococcal serotype 5 and 4, although the response in case of serotype 5 is stronger than serotype 4, suggesting a predominant serotype-specific bias.

S. mitis Serotype 5 Generates Enhanced Th17 Responses to Pneumococcal Infection

Th, particularly Th1 and Th17, responses play a crucial role in protective immunity to pneumococcal lung infections (16, 17). We have previously reported that mucosal vaccination with *S. mitis* triggers local IL-17A/Th17 immunity in the respiratory tract of mice (13). In the present study, we used *S. mitis* serotype 5 to assess its ability to generate major T helper cell responses – Th1, Th2, and Th17 – at the mucosal and peripheral tissues. First, we examined the cytokine profile of local tissues following immunization of mice with *S. mitis* serotype 5 followed by lung infection with pneumococcal serotype 5. The immunized mice produced large quantities of IL-17A (Th17) in the nasal wash, BALF, and lungs compared with mice receiving PBS (**Figure 3**). However, IFN-γ (Th1) and IL-4 (Th2) cytokine levels were not statistically different between the immunized and control groups (**Figure 3**). In order to acquire knowledge on antigen-specific T cell responses, we restimulated the mediastinal lymph node cells from the immunized mice with killed *S. mitis* serotype 5, *S.*

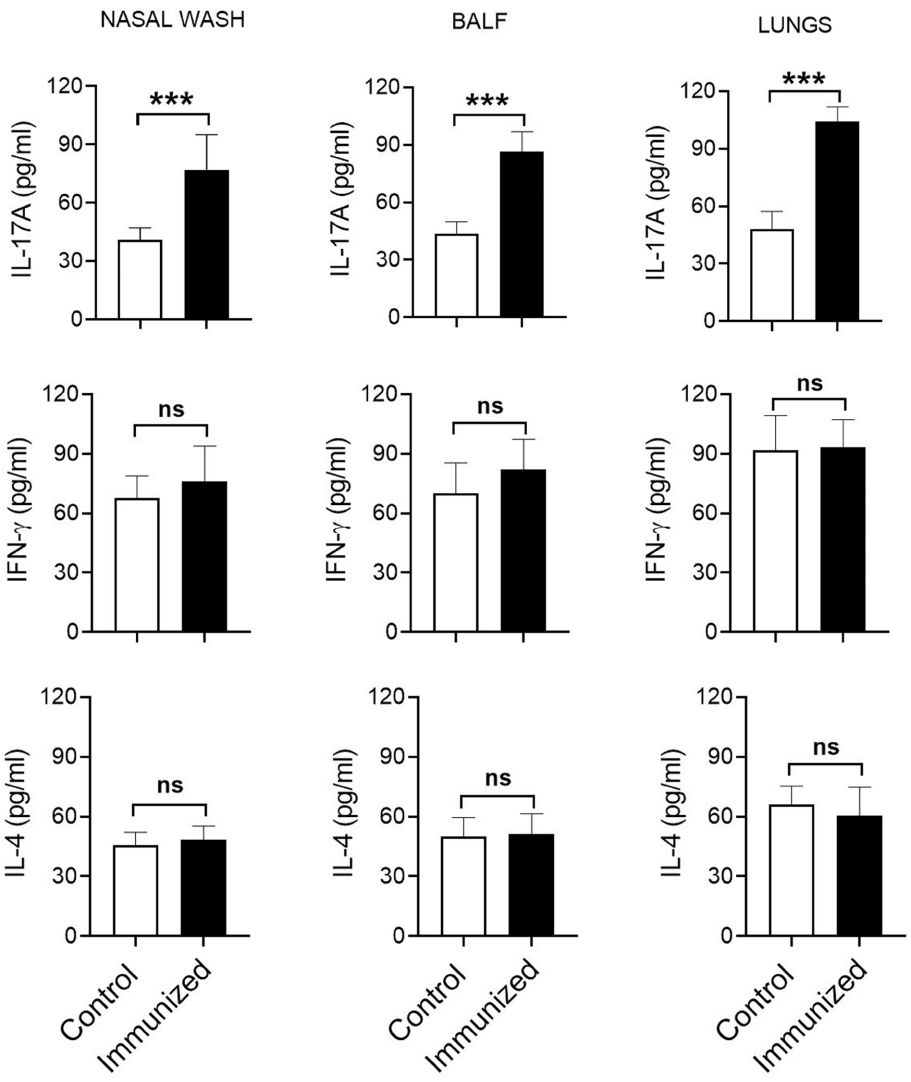

FIGURE 3 | Cytokine responses in the respiratory tract after immunization. Nasal wash, BALF, and lungs were collected from the immunized mice subjected to pneumococcal challenge infection, and the cytokine levels were measured using ELISA. Data are shown as mean ± SD and pooled from the results of two independent experiments with 4 mice in each group. ***p<0.001. Unpaired student's *t* test. ns, not significant.

pneumoniae 5 or *S. pneumoniae* 4 and measured the cytokine responses. The lymph nodes from immunized mice produced higher levels of Th17, but not Th1 and Th2, cytokines compared with control mice (**Figure 4A**). In immunized mice, the lymph nodes cells restimulated with *S. mitis* serotype 5, *S. pneumoniae* 5 or *S. pneumoniae* 4 secreted similar levels of Th17 cytokines, which were higher than in the control mice (**Figure 4A**). Then, we investigated the antigen-specific cytokine production pattern at the peripheral tissues (spleen) in response to restimulation with *S. mitis* serotype 5, *S. pneumoniae* 5 or *S. pneumoniae* 4. In line with the findings from the lymph node cell restimulation (**Figure 4A**), we found that Th17, but not Th1 and Th2, cytokine levels produced by the splenocytes restimulated with *S. mitis* serotype 5, *S. pneumoniae* 5 or *S. pneumoniae* 4 were significantly increased compared with the cytokine levels in control mice (**Figure 4B**). Thus, these findings indicate that mucosal vaccination with *S. mitis* serotype 5 induces Th17 immunity both at the local and peripheral levels.

DISCUSSION

The rare occurrence of serotype 5 invasive disease in the United States and Kenya has led to the hypothesis that the expression of serotype 5 capsule by non-pneumococcal Mitis group streptococci, including *S. mitis*, may confer cross-species immunity (18–20). Our present study supports this hypothesis because intranasal immunization of mice with *S. mitis* serotype 5 triggered protective immunity against nasal colonization by and lung infection with *S. pneumoniae* serotype 5. Our data show that the mice immunized with *S. mitis* serotype 5 exhibited significantly lower pneumococcal loads in the nasal wash, BALF, and lungs following challenge with *S. pneumoniae* 5. Of note, the pneumococcal loads in the immunized mice were around 2-3 times lower in the BALF and lungs, and 50% of the immunized ones had nil pneumococcal CFU in the BALF. This protective efficacy is higher than what has been demonstrated in similar mouse models where mice intranasally inoculated with *S. pneumoniae* serotype 6 (BHN418 6B) received a challenge with

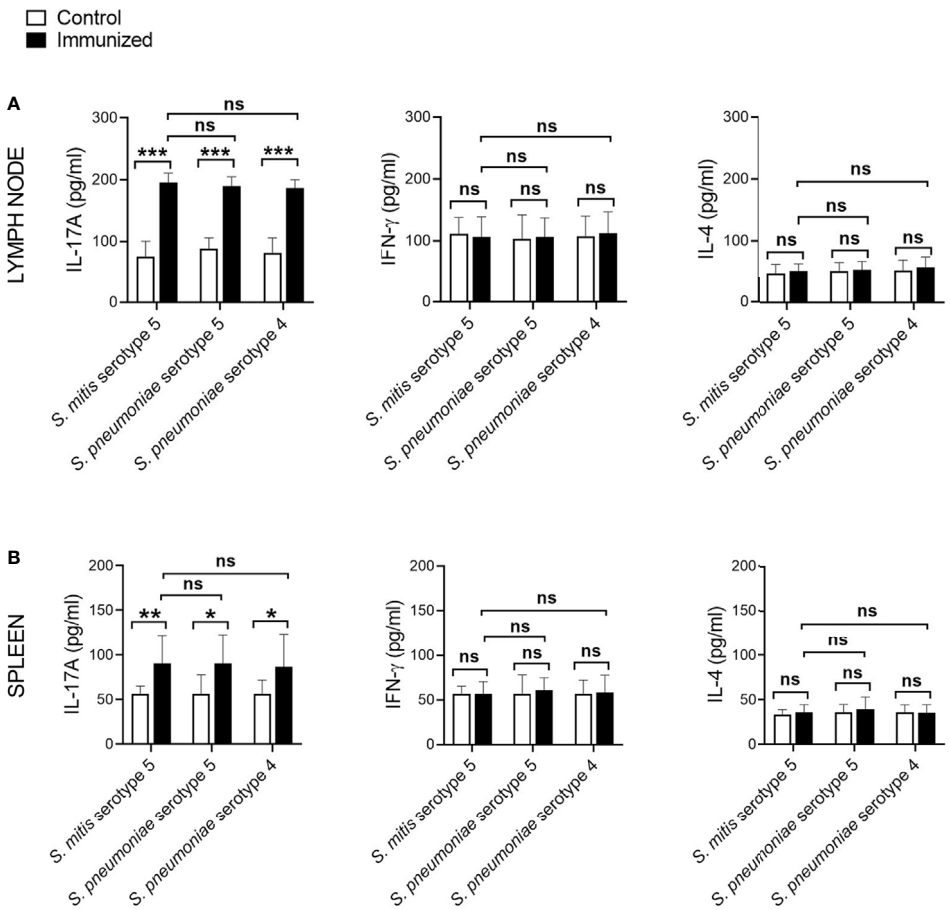

FIGURE 4 | Antigen-specific cytokine production by the lymph node cells and splenocytes following immunization. The mediastinal lymph node cells and splenocytes from the *S. mitis* serotype 5-immunized or control mice subjected to pneumococcal challenge were restimulated with killed *S. mitis* serotype 5, *S. pneumoniae* 5 or *S. pneumoniae* 4 for 72 hours, and the cytokine levels (IL-17A, IFN-γ, and IL-4) in the culture of **(A)** lymph node cells and **(B)** splenocytes were measured by ELISA. Data are shown as mean ± SD and pooled from the results of two independent experiments with 4 mice in each group. *p<0.05; **p<0.01; ***p<0.001. Unpaired student's *t* test. ns, not significant.

homologous pneumococci (21). Furthermore, *S. mitis* serotype 5 elicited robust IgG and IgA immune responses locally and systemically, which were predominantly directed against the serotype 5 capsule. It is however important to mention that *S. mitis* serotype 5 also elicited IgG/IgA antibody levels that were reactive to *S. pneumoniae* serotype 4, suggesting a serotype-independent immune response. Recently, we showed that intranasal immunization of mice with live *S. mitis* induces serotype-independent protection against infections with pneumococcal serotypes 2 and 4, which was associated with enhanced IgG and IgA responses (13). Likewise, vaccination with attenuated *S. pneumoniae* serotype 4 provided serotype-independent protection against invasive pneumococcal infections (22). The reason behind this serotype-independent immune response could be explained by the similarity between the nature of capsular and protein antigens of *S. mitis* and *S. pneumoniae*. In addition to immunogenic capsular antigens, *S. pneumoniae* possesses certain protein antigens, such as choline-binding protein D (CbpD), which are highly conserved between *S. mitis* and *S. pneumoniae* (12). Thus, the antibody responses ensued due to *S. mitis* serotype 5 in this study may be generated against capsular and protein antigens of *S. pneumoniae* serotype 5, and protein antigens of *S. pneumoniae* serotype 4.

Our current study shows that *S. mitis* serotype 5 vaccination exerts an increased antigen-specific IL-17A/Th17 immune response at the mucosal (respiratory tract) and peripheral (spleen) tissues, which is critical for protective immunity to pneumococcal lung infections (15). However, the IL-17A/Th17 responses against *S. pneumoniae* serotype 5 and *S. pneumoniae* 4 were similar, suggesting that these responses are induced against shared protein antigens between *S. mitis* serotype 5 and *S. pneumoniae* serotypes 4 and 5. This is in accordance with our previous studies where *S. mitis*-specific human Th17 cells showed cross-reactivity with *S. pneumoniae* (11), and that mice immunized with *S. mitis* promoted Th17 immunity (13). However, it remains to be ascertained whether *S. mitis* serotype 5-induced IL-17A/Th17 immunity plays a direct role in protection against pneumococcal infection, which will be addressed in our future studies by using IL-17 knockout mice. It is notable here that although Th responses, especially Th1, contribute to host defense against pneumococcal lung infection (16, 17), we failed to see any difference in Th1 (IFN-γ) and Th2 (IL-4) cytokine levels between the immunized and control groups at 24 hours after the last *S. mitis* serotype 5 immunization. Similar observations were found in our previous study where the Th response was assessed at day 7 after immunization with the *S. mitis* type strain (13). Moreover, a previous study showed that prior nasopharyngeal colonization of mice by *S. pneumoniae* resulted in significant increases in the BALF levels of Th1 cytokine (TNF-α) at 4 hours, but not at 18 hours, following pneumococcal challenge (23). Therefore, in the present study, the reason as to why we did not find difference in Th1/Th2 cytokine levels could be attributed to the time point (24 hours post-immunization) chosen to analyze the cytokines. Future studies need to assess the cytokine levels at various time points following immunization.

It is also important to discuss how relevant our findings from a mouse model are for evaluation of human-commensal effects as many gut commensals induce tolerance in the natural host (24). Previous studies in humans have shown that there exists a humoral cross-reactivity between *S. pneumoniae* and *S. mitis* (12, 25, 26), and that intranasal inoculation of adult humans with the oral streptococcal commensals induce protective immunity against otitis media caused by bacterial pathogens, including *S. pneumoniae* (27). Furthermore, inoculation with the nasopharyngeal commensal *Neisseria lactamica* in healthy individuals intranasally elicits cross-reactive systemic opsonophagocytic antibodies to the pathogen *Neisseria meningitidis* (28). Thus, these human studies indicate an important role for the oral/nasopharyngeal commensals in inducing protective immunity against respiratory pathogens. In the times to come, we plan to perform a study to investigate the impact of *S. mitis* colonization/immunization on incidence of pneumococcal carriage/infection.

In conclusion, our present study demonstrates that mucosal vaccination with live *S. mitis* serotype 5 protects against infection by *S. pneumoniae* serotype 5, and induces strong IgG/IgA and Th17 responses against pneumococcal infection at the local and systemic/peripheral tissues. Moreover, the IgG/IgA antibody levels reactive to *S. mitis* serotype 5 or *S. pneumoniae* serotype 5 were higher than the levels reactive to *S. pneumoniae* serotype 4, indicating a serotype-specific response. These findings provide significant insights into how naturally occurring commensal streptococci that express pneumococcal serotype capsule can be exploited to generate protective immunity against pneumococcal infections.

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 authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Norwegian Food Safety Authority, Oslo, Norway (Project license number FOTS – 22302).

AUTHOR CONTRIBUTIONS

SS designed research studies, conducted experiments, acquired and analyzed data, and wrote the paper. HÅ conducted experiments, acquired, and analyzed data. FP designed research studies, analyzed data, and wrote the paper. 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.2021.676488/full#supplementary-material

REFERENCES

1. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. *Lancet* (2009) 374:893–902. doi: 10.1016/S0140-6736(09)61204-6
2. Weiser JN, Ferreira DM, Paton JC. Streptococcus pneumoniae: transmission, colonization and invasion. *Nat Rev Microbiol* (2018) 16:354–67. doi: 10.1038/s41579-018-0001-8
3. Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, Bassani DG, et al. Global, regional, and national causes of child mortality in 2008: a systematic analysis. *Lancet* (2010) 375:1969–87. doi: 10.1016/S0140-6736(10)60549-1
4. Geno KA, Gilbert GL, Song JY, Skovsted IC, Klugman KP, Jones C, et al. Pneumococcal capsules and their types: past, present, and future. *Clin Microbiol Rev* (2015) 28:871–99. doi: 10.1128/CMR.00024-15
5. Jochems SP, Weiser JN, Malley R, Ferreira DM. The immunological mechanisms that control pneumococcal carriage. *PLoS Pathog* (2017) 13(12):e1006665. doi: 10.1371/journal.ppat.1006665
6. Pollard AJ, Perrett KP, Beverley PC. Maintaining protection against invasive bacteria with protein-polysaccharide conjugate vaccines. *Nat Rev Immunol* (2009) 9:213–20. doi: 10.1038/nri2494
7. Brueggemann AB, Peto TE, Crook DW, Butler JC, Kristinsson KG, Spratt BG. Temporal and geographic stability of the serogroup-specific invasive disease potential of Streptococcus pneumoniae in children. *J Infect Dis* (2004) 190:1203–11. doi: 10.1086/423820
8. Hausdorff WP. The roles of pneumococcal serotypes 1 and 5 in paediatric invasive disease. *Vaccine* (2007) 25:2406–12. doi: 10.1016/j.vaccine.2006.09.009
9. Frandsen EV, Pedrazzoli V, Kilian M. Ecology of viridans streptococci in the oral cavity and pharynx. *Oral Microbiol Immunol* (1991) 6:129–33. doi: 10.1111/j.1399-302X.1991.tb00466.x
10. Bek-Thomsen M, Tettelin H, Hance I, Nelson KE, Kilian M. Population diversity and dynamics of Streptococcus mitis, Streptococcus oralis, and Streptococcus infantis in the upper respiratory tracts of adults, determined by a nonculture strategy. *Infect Immun* (2008) 76:1889–96. doi: 10.1128/IAI.01511-07
11. Engen SA, Rukke HV, Becattini S, Jarrossay D, Blix IJ, Petersen FC, et al. The oral commensal streptococcus mitis shows a mixed memory Th cell signature that is similar to and cross-reactive with streptococcus pneumoniae. *PLoS One* (2014) 9(8):e104306. doi: 10.1371/journal.pone.0104306
12. Shekhar S, Khan R, Ferreira DM, Mitsi E, German E, Rorvik GH, et al. Antibodies reactive to commensal *Streptococcus mitis* show cross-reactivity with virulent *Streptococcus pneumoniae* serotypes. *Front Immunol* (2018) 9:747. doi: 10.3389/fimmu.2018.00747
13. Shekhar S, Khan R, Schenck K, Petersen FC. Intranasal immunization with the commensal Streptococcus mitis confers protective immunity against pneumococcal lung infection. *Appl Environ Microbiol* (2019) 85(6):e02235-18. doi: 10.1128/AEM.02235-18
14. Pimenta F, Gertz RE, Park SH, Kim E, Moura I, Milucky J, et al. Streptococcus infantis, Streptococcus mitis, and Streptococcus oralis strains with highly similar cps5 loci and antigenic relatedness to serotype 5 Pneumococci. *Front Microbiol* (2019) 9:3199. doi: 10.3389/fmicb.2018.03199
15. Wilson R, Cohen JM, Jose RJ, de Vogel C, Baxendale H, Brown JS. Protection against streptococcus pneumoniae lung infection after nasopharyngeal colonization requires both humoral and cellular immune responses. *Mucosal Immunol* (2015) 8:627–39. doi: 10.1038/mi.2014.95
16. Pido-Lopez J, Kwok WW, Mitchell TJ, Heyderman RS, Williams NA. Acquisition of pneumococci specific effector and regulatory cd4(+) t cells localising within human upper respiratory-tract mucosal lymphoid tissue. *PLoS Pathog* (2011) 7(12):e1002396. doi: 10.1371/journal.ppat.1002396
17. Wright AK, Bangert M, Gritzfeld JF, Ferreira DM, Jambo KC, Wright AD, et al. Experimental human pneumococcal carriage augments IL-17A-dependent T-cell defence of the lung. *PLoS Pathog* (2013) 9:e1003274. doi: 10.1371/journal.ppat.1003274
18. Pilishvili T, Lexau C, Farley MM, Hadler J, Harrison LH, Bennett NM, et al. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. *J Infect Dis* (2010) 201:32–41. doi: 10.1086/648593
19. Sharma D, Baughman W, Holst A, Thomas S, Jackson D, Carvalho MD, et al. Pneumococcal carriage and invasive disease in children before introduction of the 13-valent conjugate vaccine: comparison with the era before 7-valent conjugate vaccine. *Pediatr Infect Dis J* (2013) 32:E45–53. doi: 10.1097/INF.0b013e3182788fdd
20. Desai AP, Sharma D, Crispell EK, Baughman W, Thomas S, Tunali A, et al. Decline in pneumococcal nasopharyngeal carriage of vaccine serotypes after the introduction of the 13-valent pneumococcal conjugate vaccine in children in Atlanta, Georgia. *Pediatr Infect Dis J* (2015) 34:1168–74. doi: 10.1097/INF.0000000000000849
21. Ramos-Sevillano E, Ercoli G, Felgner P, Ramiro de Assis R, Nakajima R, Goldblatt D, et al. Preclinical development of virulence attenuated streptococcus pneumoniae strains able to enhance protective immunity against pneumococcal infection. *Am J Respir Crit Care Med* (2020). doi: 10.1164/rccm.202011-4161LE
22. Jang AY, Ahn KB, Zhi Y, Ji HJ, Zhang J, Han SH, et al. Serotype-independent protection against invasive pneumococcal infections conferred by live vaccine with lgt deletion. *Front Immunol* (2019) 10:1212. doi: 10.3389/fimmu.2019.01212
23. Cohen JM, Khandavilli S, Camberlein E, Hyams C, Baxendale HE, Brown JS. Protective contributions against invasive streptococcus pneumoniae pneumonia of antibody and Th17-cell responses to nasopharyngeal colonisation. *PLoS One* (2011) 6(10):e25558. doi: 10.1371/journal.pone.0025558
24. Chervonsky AV. Intestinal commensals: influence on immune system and tolerance to pathogens. *Curr Opin Immunol* (2012) 24:255–60. doi: 10.1016/j.coi.2012.03.002
25. Wilson R, Cohen JM, Reglinski M, Jose RJ, Chan WY, Marshall H, et al. Naturally acquired human immunity to pneumococcus is dependent on antibody to protein antigens. *PLoS Pathog* (2017) 13(1):e1006137. doi: 10.1371/journal.ppat.1006137
26. Khan R, Petersen FC, Shekhar S. Commensal bacteria: an emerging player in defense against respiratory pathogens. *Front Immunol* (2019) 10:1203. doi: 10.3389/fimmu.2019.01203
27. La Mantia I, Varricchio A, Ciprandi G. Bacteriotherapy with Streptococcus salivarius 24SMB and Streptococcus oralis 89a nasal spray for preventing recurrent acute otitis media in children: a real-life clinical experience. *Int J Gen Med* (2017) 10:171–5. doi: 10.2147/IJGM.S137614
28. Evans CM, Pratt CB, Matheson M, Vaughan TE, Findlow J, Borrow R, et al. Nasopharyngeal colonization by Neisseria lactamica and induction of protective immunity against Neisseria meningitidis. *Clin Infect Dis* (2011) 52:70–7. doi: 10.1093/cid/ciq065

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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Compositional Data Analysis of Periodontal Disease Microbial Communities

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Periodontal disease (PD) is a chronic, progressive polymicrobial disease that induces a strong host immune response. Culture-independent methods, such as next-generation sequencing (NGS) of bacteria 16S amplicon and shotgun metagenomic libraries, have greatly expanded our understanding of PD biodiversity, identified novel PD microbial associations, and shown that PD biodiversity increases with pocket depth. NGS studies have also found PD communities to be highly host-specific in terms of both biodiversity and the response of microbial communities to periodontal treatment. As with most microbiome work, the majority of PD microbiome studies use standard data normalization procedures that do not account for the compositional nature of NGS microbiome data. Here, we apply recently developed compositional data analysis (CoDA) approaches and software tools to reanalyze multiomics (16S, metagenomics, and metabolomics) data generated from previously published periodontal disease studies. CoDA methods, such as centered log-ratio (clr) transformation, compensate for the compositional nature of these data, which can not only remove spurious correlations but also allows for the identification of novel associations between microbial features and disease conditions. We validated many of the studies' original findings, but also identified new features associated with periodontal disease, including the genera *Schwartzia* and *Aerococcus* and the cytokine C-reactive protein (CRP). Furthermore, our network analysis revealed a lower connectivity among taxa in deeper periodontal pockets, potentially indicative of a more "random" microbiome. Our findings illustrate the utility of CoDA techniques in multiomics compositional data analysis of the oral microbiome.

Keywords: periodontal disease, CLR, compositional data analysis, microbiome, oral microbiome, C-reactive protein

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INTRODUCTION

Periodontal disease (PD) manifests as bacterial biofilms (plaque) that lead to gum inflammation, recession, and, in later stages, degradation of the bone and tooth loss. Despite the prevalence of the disease, which affects over 45% of United States adults, the precise role of the oral microbiome in the progression of PD remains elusive (Eke et al., 2012). Prior to the development of next-generation sequencing (NGS) technologies, a cluster of three species deemed the "red complex," consisting of *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, was found to be associated with the PD clinical factors gum pocket depth and bleeding (Socransky et al., 1998).

While some individual species of the oral microbiome contributing to PD, such as the members of the red complex, have been studied extensively (Lamont and Jenkinson, 1998), the presence of these specific species is not enough to explain the occurrence of PD (Ximénez-Fyvie et al., 2000). NGS technologies have revealed greater diversity of the oral microbiome and a complex relationship between microbiome composition and periodontal disease states, including an association between increasing microbial diversity and pocket depth (Kroes et al., 1999; Paster et al., 2001; Faveri et al., 2008; Griffen et al., 2012). Analysis of periodontal disease metagenomes has also revealed a novel bacterium strongly associated with the red complex and periodontal disease (Torres et al., 2019). Furthermore, high inter-patient diversity of the oral microbiome complicates deciphering the relationship between periodontal treatments or changes in disease state on the associated microbiome (Kumar et al., 2006; Schwarzberg et al., 2014; Califf et al., 2017).

While NGS technologies illuminate a great deal of information about the oral microbiome, most microbiome analyses ignore the compositional structure of NGS microbiome data, which presents problems in statistical and biological interpretation. Microbiome data are compositional for two main reasons. First, sequencing only captures a proportion of the microbes in a sample, so the counts of taxa in each sample are relative rather than absolute. As the measurement of one taxon increases, the measurement of another taxon must decrease regardless of whether its absolute abundance is actually lower. Second, as the count total obtained in a run of NGS sequencing is capped by sequencing depth limitations, each sample size is different, rendering the counts of taxa between samples incomparable. Common normalization methods for microbiome data, such as rarefaction, transcripts per million, and library size normalizations, attempt to make samples with different library sizes comparable, but generate proportional data still constrained by its relative nature (Gloor et al., 2017). Metabolomics, another data type that is commonly used in conjunction with microbiome analysis, is also relative in nature and therefore compositional. Furthermore, the integration of multiomics data, or different "omics" datasets like proteomics, metabolomics, and metagenomics, from the same sample is challenging due to the different scales with which these data are measured.

Most statistical tests assume that the sample data exist in real space, where Euclidian geometry and distance formulas can be used to describe the distance between points. However, compositional data exist in a space known as the simplex where dimensions are arbitrary and values are subject to spurious correlations (Aitchison, 1982; Gloor et al., 2017). Compositional data analysis (CoDA) approaches have been developed to deal with these constraints of compositional data. One method gaining traction is the centered log-ratio (clr) transformation, which recasts relative count data with respect to the sample's geometric mean and creates scale-invariant data in Euclidian space where the use of multivariate statistical methods is valid (Gloor et al., 2017; Quinn et al., 2018). We recently showed that analyzing clr-transformed compositional datasets can reveal novel relationships, allow better discrimination between variables, and facilitate the integration of multiomics 16S, internal transcribed spacer (ITS), and metabolomic datasets (Sisk-Hackworth and Kelley, 2020).

In this work, we applied CoDA approaches, namely, clr transformation prior to standard methods such as non-metric multidimensional scaling (NMDS) ordination, Spearman's correlation, multiomics structure correlation, beta dispersion, random forest, and network analysis, as well as the log-ratio balance method used in the R package selbal (Rivera-Pinto et al., 2018), to 16S, metagenomic, cytokine, and metabolomic datasets from prior studies of patients with periodontal disease before and after treatments. By reanalyzing these data with a CoDA approach, we integrated these multiomics datasets to reveal patterns and correlations between the disease state, microbes, metabolites, and cytokines, in addition to the relationships between community structure and disease state not identified with standard normalization methods.

MATERIALS AND METHODS

Study Descriptions

This study incorporated data from two separate studies. The standard periodontal treatment (PT) study consisted of patients with periodontal disease and investigated the biofilms of periodontal pockets through 16S sequences, metagenomic sequences, and serum cytokine levels before and after standard periodontal treatments (Schwarzberg et al., 2014; Delange et al., 2018; Vijay Kumar et al., 2018). A total of 21 males and 38 females with an average age of 29 years were recruited from an American Indian/Alaska Native population in Southern California for the PT study. Eight patients had mild periodontitis (pocket depth less than 3 mm), 40 had moderate periodontitis (3–6 mm), and 11 had severe periodontitis (pocket depth over 6 mm). The second study measured pocket metabolites, 16S sequences, metagenomic sequences, and the serum cytokine levels of patients before and after treatments with 0.25% sodium hypochlorite (SHT) (Califf et al., 2017). For this study, 19 males and 15 females with an average age of 41 years were recruited among patients of the Ostrow School of Dentistry at the University of Southern California. In the SHT study, periodontal pocket depths ranged from 3 to 12 mm, while pocket depths in the PT ranged from 1.3 to 3.8 mm. The disease classes for the SHT study were separated into class "A" (pocket depth up to 6 mm), class "B" (pocket depth between 6 and 8 mm), and class "C" (pocket depth over 8 mm). Further details on the patient populations can be found in Schwarzberg et al. (2014) and Delange et al. (2018) for the PT study and in Califf et al. (2017) for the SHT study.

PT Study Data

The original PT data contained 76 samples of 247 16S operational taxonomic units (OTUs), 144 samples of six cytokine inflammatory markers, and 23 samples of 3,830 bacterial metagenomic OTUs. The 16S ribosomal RNA (rRNA) sequences and the mapping file from in this study are accessible at: http://dx.doi.org/10.6084/m9.figshare.855613 and http://dx.doi.org/10.6084/m9.figshare.855612.

The serum cytokine data, raw reads from the 16S rRNA sequences, and metagenomic OTUs, classified by Kraken, were published previously (Delange et al., 2018; Vijay Kumar et al., 2018; Torres et al., 2019). Details on the study population, sampling, disease classification, and cytokine identification can be found in previously published papers (Schwarzberg et al., 2014; Delange et al., 2018; Vijay Kumar et al., 2018; Torres et al., 2019).

SHT Study Data

The SHT study contained 286 samples of 773 16S OTUs, 215 samples of 914 tandem mass spectrometry (MS/MS) features, and 24 samples of 3,770 bacterial metagenomic features. The 16S rRNA sequences used in this study were accessed through the European Nucleotide Archive under project PRJEB19122 (Califf et al., 2017). Metabolite data from tandem mass spectrometry were downloaded from the online MassIVE repository of the GNPS database under MassIVE ID number MSV000078894. Metagenomic sequence libraries, generated from 24 subgingival samples from the SHT study patients and classified *via* Kraken, were obtained from Dr. Pedro Torres (Torres et al., 2019).

16S Sequence Analysis

16S sequencing data were analyzed using QIIME 2020.2 (Bolyen et al., 2019). Sequences were clustered into 100% identity using v-search OTU clustering (Rognes et al., 2016). Taxonomy was assigned to sequences using the RDP Classifier (Wang et al., 2007) retrained on Greengenes 13_5 (McDonald et al., 2012) *via* QIIME 2.

Data Reduction and Transformation

Due to computational constraints, the numbers of features in the original sequencing and metabolomic datasets were reduced for *selbal* analysis (see below). The same reduced datasets were then used for the rest of the analyses. Genera of the 16S bacterial taxa present in greater than 10% of the samples, the 181 most abundant metagenomic taxa counts, and the 65 most abundant metabolites were selected for correlation analysis. For both PT and SHT studies, samples with a NA value for pocket depth in the mapping file were removed from all analyses. For the PT data, only samples with an overall response of improved or worsened were kept for all the analyses, determined by whether pocket depth decreased or increased, respectively (Schwarzberg et al., 2014). For the SHT data, only subgingival samples with disease class "A" or "C" were used on all analyses, as class "B" contained too few samples. Disease status was classified by maximum pocket depth ("A" = up to 6 mm, "B" = 6–8 mm, and "C" = over 8 mm) (Califf et al., 2017). For both the PT and SHT studies, OTUs were summed by genus for each sample in each of the 16S and metagenomic datasets. Zero replacement was performed with the pseudo-counts method from the R package zCompositions (Palarea-Albaladejo and Martín-Fernández, 2015) version 1.3.3. clr transformation was performed separately on all datasets (not on combined "multiomics" datasets). The clr transformation was computed for each sample *j*: each feature in that sample was divided by the geometric mean of all the feature counts in the sample, then the natural log of that ratio was taken (Aitchison, 1982).

$$\mathrm{clr}\left(X_j\right) = \left[\ln\left(\frac{X_{1j}}{g(X_j)}\right), \cdots, \ln(\frac{X_{Dj}}{g(X_j)})\right]$$

where X_j is the list of features in a sample, $g(X_j)$ is the geometric mean of the features in sample X_j, X_{1j} is the first feature in a sample, and X_{Dj} is the last feature in a sample of D values. To guide the reader, we have provided a diagram of the various datasets and analyses used in this study (**Figure 1**).

NMDS Ordination Plots

clr-transformed values were used to generate the NMDS ordination plots with the R package vegan version 2.5.6 using Euclidean distances (Oksanen et al., 2019). The NMDS plots were created in R using ggplot2 version 3.2.1c (Wickham, 2016) and the samples were colored and shaped by periodontal treatment, overall response, and disease class. Permutational multivariate analysis of variance (PERMANOVA) was performed for each condition (periodontal treatment, overall response, and disease class) in every dataset (16S bacteria, cytokine, metabolites, and bacterial metagenomics) using the R package vegan with 9,999 permutations, with the p values corrected for multiple comparisons using the Benjamini–Hochberg method. The multivariate PERMANOVA test determines whether the centroid of a sample set is equal among the specified categories (e.g., periodontal treatment and disease class). The centroid was estimated using the between-sample Euclidean distances.

Beta Dispersion

Beta dispersion, which measures the distance of each sample in a category from the centroid of that category, was estimated with the R package vegan using between-sample Euclidian distances. The beta dispersion test is a multivariate test used to determine whether the dispersion of samples is equivalent among categories. The p values were adjusted using the Benjamini–Hochberg method.

Spearman's Correlations

Spearman's correlations were computed using the R package psych v1.0.67 (Revelle, 2020). For each periodontal treatment, overall response, and disease class, we computed the correlations between genera from four different combined multiomics datasets: (1) 16S bacteria and cytokine (PT); (2) 16S bacteria, cytokine, and bacterial metagenomics (PT); (3) bacteria and metabolite (SHT); and (4) bacteria, metabolite, and bacterial metagenomics (SHT). The p values were adjusted with the Bonferroni correction.

Multiomics Integration

We integrated the same datasets as in the Spearman's correlations using the DIABLO framework, a method for multiomics classification and integration, in the mixOmics R package version 6.10.8c (Rohart et al., 2017). We assessed the correlation structure at the component level for each of the three conditions on

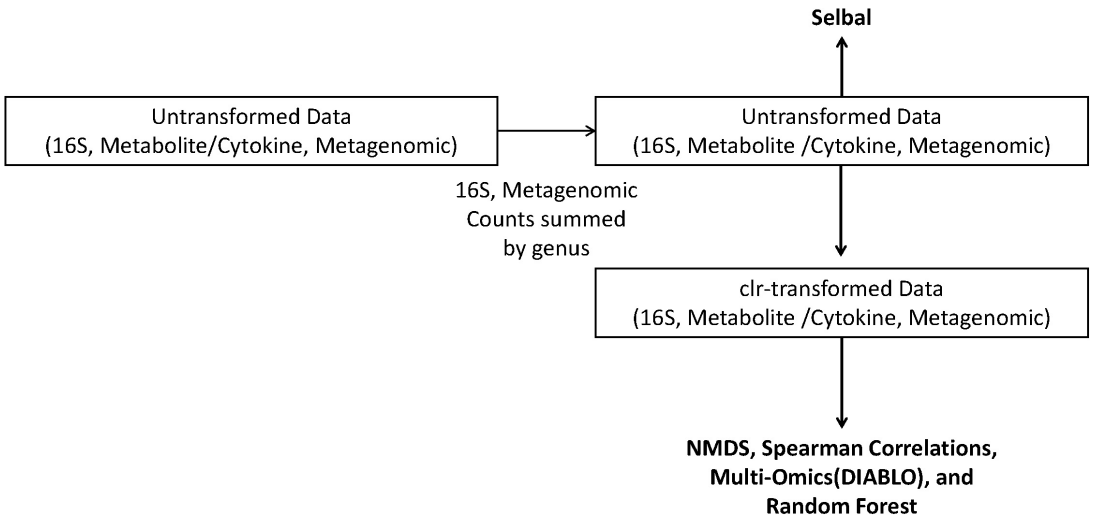

FIGURE 1 | Schematic of the analyses performed on the centered log-ratio (clr)-transformed periodontal treatment (PT) and sodium hypochlorite (SHT) data. Only untransformed data were inputted into selbal, and clr-transformed data were used to calculate Spearman's correlations, non-metric multidimensional scaling (NMDS), and DIABLO analyses.

their respective dataset: periodontal treatment, overall response, and disease class.

Microbial Balances

We identified differentially abundant taxa, metabolites, and cytokines using the R package selbal version 0.1, a compositional data analysis method that detects microbial signatures between different sample types by identifying the smallest number of differentially abundant taxa that is predictive of sample condition. Raw measurements of cytokines, metabolites, 16S, and metagenomic taxa summed by genus were inputted to selbal, as it performs zero handling and transformation within the package. Although selbal was designed with microbial balances in mind, the method is valid for finding balances of other data types, such as metabolite and cytokine data. Furthermore, selbal only finds balances for dichotomous and continuous response variables, so we performed this analysis only for the variables periodontal treatment (dichotomous), disease class (dichotomous), and pocket depth (continuous).

Random Forest

A random forest classifier was implemented in Python using the scikit-learn package (Pedregosa et al., 2012) to identify cytokines, metabolites, and 16S genera that discriminate between pocket depth (PT) and disease class (SHT). The metrics used to analyze the random forest classifier include accuracy, out-of-bag (OOB) score, mean accuracy, and area under the curve of the receiver of components (AUC-ROC). For the PT data, the pocket depth boundary used to distinguish high and low pocket depths was 2.6 mm. For the SHT study, only disease classes "A" and "C" were used as disease class "B" had few samples.

Network Analysis

Using the R package psych, we calculated Pearson's correlations for each of the following datasets: 16S OTU, combined 16S OTU–cytokine for the PT study, and 16S for the SHT study. Correlations with a magnitude of $|0.55|$ or greater were kept; all other values were changed to zero. Using the psych package, p values were calculated for each pairwise Pearson's correlation. The correlation matrix and the p value matrix were then filtered to contain only significant correlations (those with a Bonferroni-corrected p value below 0.05). The resulting adjacency matrix was transformed into an igraph object using a function from the SpiecEasi library (Kurtz et al., 2015). Using igraph v.1.2.5 package (Csardi and Nepusz, 2006), a network was constructed from the adjacency matrix using the OTUs as nodes and the Pearson's correlation values as edge weights. Networks were constructed with nodes scaled according to the eigen centrality.

For each network, we calculated the number of nodes, edges, as well as the diameter and transitivity. Nodes represent individual genera or cytokines and edges are lines representing relationships between genera or cytokines. The diameter of a network is the shortest distance between the furthest apart nodes in a network. Transitivity, ranging from 0 to 1, measures the average connectedness of a network, with higher values signifying that a high proportion of nodes are connected to surrounding nodes, which indicates the presence of tightly connected clusters of nodes. To identify taxa that occupy important structures of the network, the R package igraph was used to calculate the eigenvector centrality (eigen centrality) and betweenness centrality. Eigen centrality identifies which highly connected nodes are connected to other highly connected nodes; these highly connected nodes therefore form most of the architecture that orders the network. Betweenness centrality represents the frequency that a node is traversed

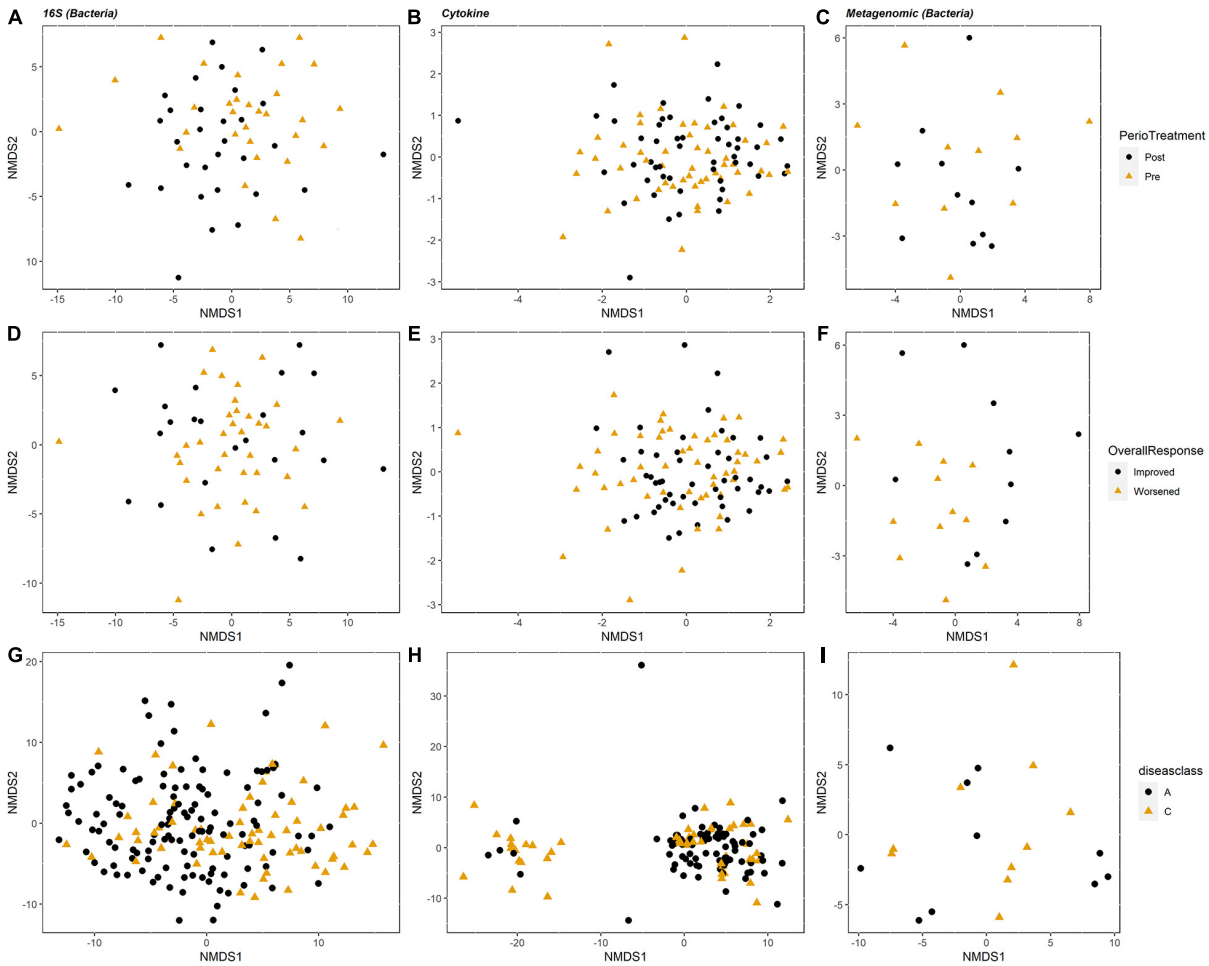

FIGURE 2 | Non-metric multidimensional scaling (NMDS) ordination plots showing clustering of the samples. For the periodontal treatment (PT) study **(A–F)**, *columns* correspond to dataset type: 16S, cytokines, and metagenomics are columns 1, 2, and 3, respectively (n = 60, 104, and 22). The *top row* **(A–C)** is colored by periodontal treatment and the *second row* **(D–F)** colored by overall response. For the sodium hypochlorite (SHT) study **(G–I)**, *columns* correspond to dataset type: 16S, metabolomics, and metagenomics are columns 1, 2, and 3, respectively (n = 209, 153, and 24). Plots are colored by disease class.

when the shortest paths in a network are calculated; high betweenness centrality indicates nodes that facilitate correlations between other nodes.

RESULTS

Beta Diversity

We used NMDS ordination to determine the clustering of samples by condition (periodontal treatment, overall response, disease class, and pocket depth) for microbes, cytokines, and metabolites for the PT and SHT study datasets. For the 16S, cytokine, and metagenomic datasets from the PT study, we did not observe clustering of samples by periodontal treatment or overall response (**Figure 2**) or pocket depth (**Supplementary Figure 1**). The most distinct separation was seen in the metabolites for disease class in the SHT study, where most of the samples in disease class "A" clustered together and the samples in class "C" split into two groups (**Figure 2H**); a similar pattern was observed in the metabolites for pocket depth (**Supplementary Figure 2**). PERMANOVA indicated a difference between periodontal treatment groups for the 16S and cytokine datasets in the PT study (p −adj = 0.0234 for both comparisons; **Table 1**) and for the 16S and metabolite datasets in the SHT study (p −adj = 0.0.0012 and 0.006, respectively; **Table 1**). Analysis of beta dispersion showed no differences between the periodontal treatment or overall response groups in the PT study or by disease class in the SHT class (**Supplementary Figure 3**).

Spearman's Correlations

clr transformation reduces spurious correlations in compositional data, such as microbiome and metabolome data, and allows the application of statistical methods such as Spearman's correlation (Quinn and Erb, 2019). We applied Spearman's correlation to analyze the relationships between the multiomics datasets from both the PT and SHT studies. Most correlations between the combined multiomics datasets

TABLE 1 | PERMANOVA results (9,999 permutations) for centered log-ratio (clr)-transformed periodontal treatment (PT) 16S, cytokine, and metagenomic datasets and sodium hypochlorite (SHT) 16S, metabolite, and metagenomic datasets.

Dataset	Variable	R^2	p	p – adj[a]
PT study				
16S	Periodontal treatment	0.0343	0.0053	0.0234
	Overall response	0.0129	0.7848	0.7848
Cytokine	Periodontal treatment	0.0343	0.0078	0.0234
	Overall response	0.0175	0.1152	0.1683
Metagenomic	Periodontal treatment	0.0687	0.1403	0.1683
	Overall response	0.0972	0.0293	0.0586
SHT study				
16S	Disease class	0.032	0.0004	0.001
Metabolite	Disease class	0.028	0.0040	0.006
Metagenomic	Disease class	0.044	0.5482	0.548

[a] *Benjamini–Hochberg corrected for multiple comparisons.*

were within the same datasets (e.g., bacteria to bacteria), while few between-omics correlations (e.g., bacteria to cytokines) were detected. For the PT datasets, no significant ($p < 0.05$) bacteria–cytokine correlations were observed, except in the posttreatment samples that had worsened. In these samples, *Prevotella* was strongly correlated ($R^2 = 0.808$) with interleukin (IL)-1 (**Supplementary Figure 4**).

In the SHT study, we observed many significant correlations among bacteria and metabolites when the datasets for samples of disease classes "A" and "C" were combined; *Acinetobacter*, *Rubrivivax*, and *Treponema* were positively correlated with six metabolites, while *Desulfovibrio*, *Paludibacter*, *Peptococcus*, *TG5*, and *Treponema* were negatively correlated with six different metabolites (**Supplementary Figure 5A**). In samples that were only disease class "A," *Olsenella* and *Atopobium* were positively correlated with two metabolites, while *Treponema* was negatively correlated with one metabolite (**Supplementary Figure 5B**). No bacterial–metabolite correlations were observed in samples that were only disease class "C."

Multiomics Integration

Using DIABLO, we found that the correlation structure between the 16S and cytokine datasets in the PT study was better when the overall response (improved or worsened) variable was included than when the time (pre *vs*. post) variable was incorporated (**Figures 3A,C**). When metagenomic data were included in the multiomics correlation (excluding samples that did not get the metagenome sequenced), the correlation structure did not change dramatically, but there was greater discrimination of samples by overall response (**Figures 3B,D**). For the SHT study, the metabolite and 16S combined datasets strongly distinguished between disease class, but the correlation structure was low (**Figure 3E**). When metagenomic data were included in the multiomics correlation (excluding samples that did not get the metagenome sequenced), the correlation structure greatly increased, but this may be an artifact of the severely reduced sample size (**Figure 3F**).

We generated Circos plots showing the correlations between the "omics" datasets using the DIABLO correlation structure. In the PT study, IL-6/IL-10 were strongly negatively correlated with *Treponema* and *Schwartzia* (**Figure 4A**). In the SHT study, TG5 and *Treponema* were strongly correlated with many unidentified metabolites (**Figure 4B**).

Microbial Balances

Using selbal, we analyzed the differentially abundant genera, cytokines, and metabolites between different conditions. In the PT study, selbal identified many bacterial genera predictive of pretreatment samples, *Desulfobulbus*, *Bulleidia*, and *Hylemonella*, while *Abiotrophia* and *Haemophilus* were more predictive of posttreatment samples (**Table 2** and **Supplementary Figure 6A**). For cytokines, selbal identified IL-6 as the most predictive of pretreatment samples and IL-6/IL-10 as the most predictive of posttreatment samples (**Table 2** and **Supplementary Figure 5B**). From the metagenomic dataset, selbal identified that *Prevotella* predicted pretreatment and *Haemophilus* predicted posttreatment samples (**Table 2** and **Supplementary Figure 5C**). In distinguishing overall response among the datasets, selbal identified *Desulfobulbus*, IL-6, and *Burkholderia* as more predictive in samples that improved and *Peptococcus*, C-reactive protein (CRP), and *Coryebacterium* as more predictive in samples that worsened (**Table 2** and **Supplementary Figures 6D–F**). We then analyzed pre- and posttreatment balances for samples that improved and samples that worsened. In pretreatment samples that ended up improving, *Campylobacter*, *Bulleidia*, *Treponema*, IL-10, and *Actinomyces* were more relatively abundant, while in improved samples posttreatment *Peptococcus*, IL-1I(^2), and *Rothia* were relatively abundant (**Table 2** and **Supplementary Figures 7A–C**). In pretreatment samples that later worsened, *TG5*, CRP, and *Veillonella* were more relatively abundant, while in posttreatment samples that had worsened, *Fusobacterium*, IL-6/IL-10, and *Haemophilus* were more relatively abundant (**Table 2** and **Supplementary Figures 7D–F**).

We also used selbal to explore which features' relative abundance changed with the sum of all pocket depths. Within the improved samples, selbal identified *Selenomonas* and IL-10 as more associated with shallow pocket depths and *Filifactor*, IL-1I(^2), and *Porphyromonas* as more associated with deeper pocket depths (**Table 2** and **Supplementary Figures 8A–C**). Within the worsened samples, *Filifactor*, CRP, and *Veillonella* were associated with shallow pocket depths, while *Paludibacter*, IL-6/IL-10, and *Bacillus* were associated with deeper pocket depths (**Table 2** and **Supplementary Figures 8D–F**).

We also used selbal to determine the metabolite and microbial balances in the SHT study 16S and metagenomic datasets for disease class and pocket depth. Both 16S and metagenomic microbial balances identified the genus *Tanerella* as more predictive for disease class "A" and *Fusobacterium* as more predictive of disease class "C" (**Table 3** and **Supplementary Figures 9A,C**). Metabolite balances were clearly identified, but the specific metabolites in the balance remain unknown (**Table 3** and **Supplementary Figures 8B,E,H**). In disease class "A" samples,

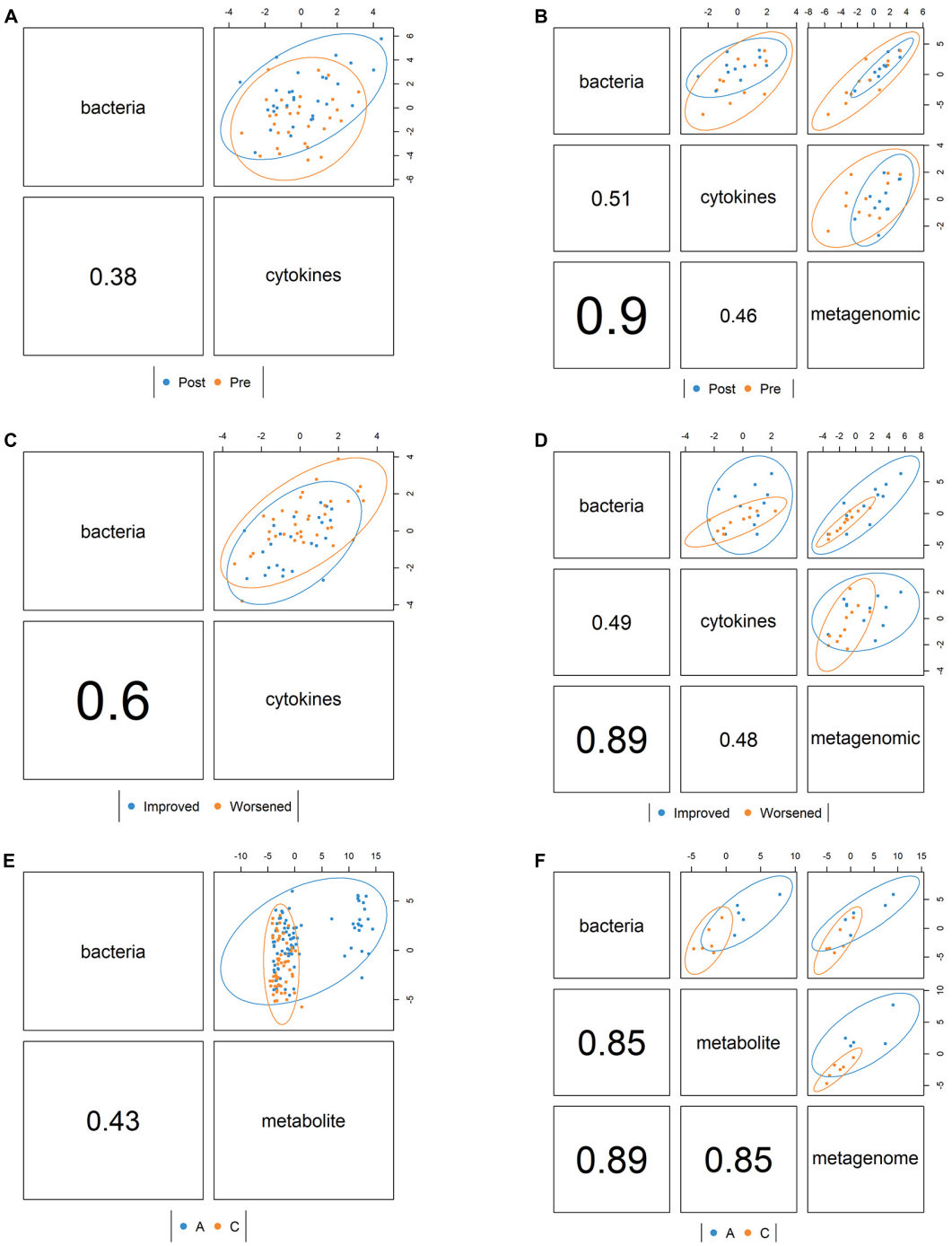

FIGURE 3 | Correlation structure between datasets as determined by the mixOmics DIABLO framework colored by pre- *vs*. posttreatment for the periodontal treatment (PT) study **(A)** bacterial 16S and cytokine datasets and **(B)** bacterial 16S, cytokine, and metagenomic datasets and also colored by whether the disease improved or worsened for **(C)** the bacterial 16S and cytokine datasets and **(D)** the bacterial 16S, cytokine, and metagenomic datasets. For the sodium hypochlorite (SHT) study, samples were colored by disease class for **(E)** the bacterial 16S and metabolic datasets and **(F)** the bacterial 16S, metabolites, and metagenomic datasets. Values indicate the between-dataset correlation structure. *Ellipses* indicate discrimination by the multiomics components between samples by condition.

selbal identified *Desulfobulbus* and *Rothia* as more predictive of shallower pocket depths and *SHD-231* and *Fusobacterium* as more predictive of deeper pocket depth (**Table 3** and **Supplementary Figures 9D,F**). For the samples in disease class "C," shallow pocket depth was more associated with *Lactobacillus* and *Parabacteroides*, while deeper pocket depth was more associated with *Desulfovibrio* and *Bacteroides* (**Table 3** and **Supplementary Figures 9G,I**).

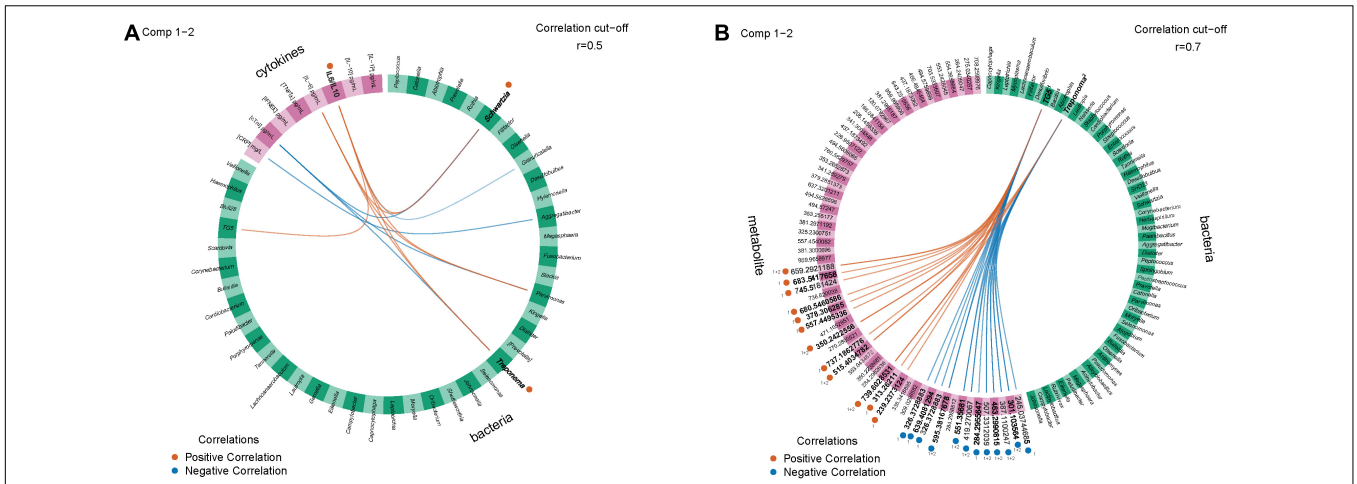

FIGURE 4 | Circos plots show 16S and other features with inter-omics links indicating positive or negative correlations with an R^2 greater than 0.5 between **(A)** the periodontal treatment (PT) study for the improved and worsened samples with the strongest correlation (0.6) for IL-6/IL-10 positively correlated with *Schwartzia* and *Treponema* and **(B)** the sodium hypochlorite (SHT) study for correlations with an R^2 greater than 0.7 for disease classes "A" and "C." *Bold labels* were the strongest correlations.

TABLE 2 | Summary of the microbial and metabolic balances generated from the periodontal treatment (PT) datasets.

Dataset	Variable	Denominator	Numerator	AUC-ROC
16S	Periodontal treatment	*Desulfobulbus*, *Bulleidia*, *Hylemonella*	*Abiotrophia*, *Haemophilus*	0.85
	Overall response	*Desulfobulbus*	*Peptococcus*	0.76
	Periodontal treatment for improved	*Campylobacter*, *Bulleidia*, *Treponema*	*Haemophilus*	0.83
	Periodontal treatment for worsened	*TG5*	*Fusobacterium*	0.76
	Pocket depth for improved	*Selenomonas*	*Filifactor*	0.73
	Pocket depth for worsened	*Filifactor*	*Paludibacter*	0.23
Cytokine	Periodontal treatment	IL-6	IL-6/IL-10	0.60
	Overall response	IL-6	CRP	0.66
	Periodontal treatment for improved	IL-10	IL-1I(^2)	0.66
	Periodontal treatment for worsened	CRP	IL-6/IL-10	0.58
	Pocket depth for improved	IL-10	IL-1I(^2)	0.42
	Pocket depth for worsened	CRP	IL-6/IL-10	0.06
Metagenomic	Periodontal treatment	*Prevotella*	*Haemophilus*	0.86
	Overall response	*Burkholderia*	*Corynebacterium*	0.90
	Periodontal treatment for improved	*Actinomyces*	*Rothia*	1.00
	Periodontal treatment for worsened	*Veillonella*	*Haemophilus*	1.00
	Pocket depth for improved	*Selenomonas*	*Porphyromonas*	0.90
	Pocket depth for worsened	*Veillonella*	*Bacillus*	0.74

AUC-ROC, area under the curve of the receiver of components.

Random Forest

The random forest machine learning classifier was trained to determine how accurately pocket depth class and disease class could be predicted from 16S bacteria OTUs and cytokines for the PT study or from 16S bacteria and metabolites for the SHT study. The most important cytokines were IL-10 and CRP (**Figure 5B**). The 16S features in the SHT study most predictive of disease class changed dramatically when metabolite data were included in the random forest analysis; the only feature recognized as highly important in both classifiers was *Abiotrophia* (**Figures 5A–C**). The addition of metabolite data to the 16S data increased the AUC-ROC scores compared with the 16S by itself in the SHT study (**Table 4**). However, the inclusion of the cytokine features did not improve the AUC-ROC scores in the PT study (**Table 4**).

Network Analysis

In the samples with moderate disease conditions, the Pearson's correlation networks had approximately twice as many edges as those with gingivitis and a small diameter (**Table 5** and **Figures 6A,B**). Transitivity was not different between disease conditions, implying that both networks have similar levels of inter-nodal interactions. In the gingivitis network, seven taxa formed correlations, of which four had eigen centralities that were greater than 0.1: *Pelomonas*,

TABLE 3 | Summary of the microbial and metabolic balances generated from the sodium hypochlorite (SHT) datasets.

Dataset	Variable	Denominator	Numerator	AUC-ROC
16S	Disease class	*Capnocytophaga*	*Porphyromonas*	0.77
	Pocket depth for class A	*Desulfobulbus*	*SHD-231*	0.04
	Pocket depth for class C	*Lactobacillus*	*Desulfovibrio*	0.208
Metabolite	Disease class	301.1035645	637.32011211, 554.3658664	0.74
	Pocket depth for class A	419.270067	284.2958512	0.127
	Pocket depth for class C	245.0374468	494.5759899	0.264
Metagenomic	Disease class	*Tannerella*	*Fusobacterium*	0.88
	Pocket depth for class A	*Bacillus*	*Rothia*, *Fusobacterium*	0.892
	Pocket depth for class C	*Parabacteroides*	*Bacteroides*	0.848

AUC-ROC, area under the curve of the receiver of components.

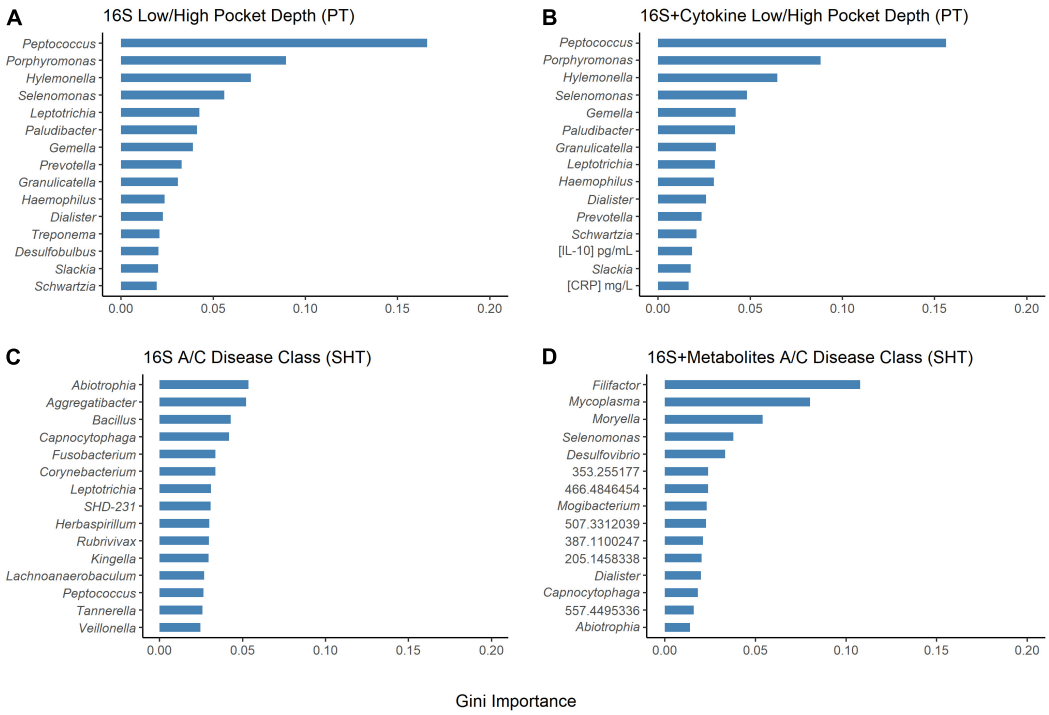

FIGURE 5 | Results of random forest Gini importance bar plots. Periodontal treatment (PT) dataset high/low periodontal disease (PD) using **(A)** 16S and **(B)** 16S and cytokines. Sodium hypochlorite (SHT) dataset A/C disease classes **(C)** 16S and **(D)** 16S and metabolites. Larger Gini importance indicates that features can resolve more nodes in decision trees with more confidence.

Thermoanaerobacterium, *Aeribacillus*, and *Ralstonia* (**Figure 6A** and **Supplementary Table 1**). The degree distribution of the gingivitis network did not strictly follow the characteristic shape of a power law distribution, but it did reveal high amounts of "low connectivity" and low amounts of "high connectivity," while the moderate network had a degree distribution that followed the power law trend much more closely (**Supplementary Figures 10A,B**).

Networks were also constructed for samples that had either shallow or deep pocket depths. Networks from the samples with shallow pockets had approximately three times as many edges as those with deep pockets (**Table 5** and **Figures 6C,D**). The networks from the samples with shallower pockets had greater transitivity than those with deep pockets. No cytokines were highly connected (high eigen centralities) within any of the networks we constructed (**Supplementary Tables 1–4**), suggesting that cytokines were not strongly correlated with many taxa. The degree distribution for these networks followed the typical shape of a power law distribution (**Supplementary Figures 10C,D**).

For the SHT study, networks were constructed with 16S data for the samples from class A and class C disease status. The network structure characteristics were similar between the two networks describing the microbiomes of patients diagnosed with different disease classes (**Table 4** and **Figures 6E,F**). The taxa with the highest eigen centralities in disease class "A" were *Streptobacillus*, *Aerococcus*, and *Arthrobacter*, while *Aerococcus*, *Massilia*, and *Gemella* had the highest eigen centralities in

TABLE 4 | Results of random forest analysis with single and combined multiomics.

Comparisons	Accuracy	OOB score	Mean accuracy	AUC-ROC
PT study				
High *vs*. low (16S)	0.63	0.65	0.59	0.59
High *vs*. low (16S + cytokine)	0.63	0.57	0.59	0.59
SHT study				
A *vs*. C (16S)	0.72	0.69	0.73	0.6
A *vs*. C (16S + metabolite)	0.76	0.71	0.74	0.7

OOB score, out-of-bag score; AUC-ROC, area under the curve of the receiver of components.

TABLE 5 | Summary statistics of the networks generated from the periodontal treatment (PT) datasets comparing 16S and cytokines based on disease severity and pocket depth and the sodium hypochlorite (SHT) 16S datasets comparing oral microbiomes with different disease classes.

Data	Condition	Vertices	Edges	Diameter	Transitivity
16S	Gingivitis	48	32	10	0.49
	Moderate	48	69	6	0.45
16S + cytokine	Gingivitis	56	42	12	0.43
	Moderate	56	78	7	0.42
16S	Shallow	48	62	7	0.55
	Deep	48	22	6	0.20
16S + cytokine	Shallow	56	70	8	0.52
	Deep	56	26	6	0.18
16S	Class A	86	130	8	0.50
	Class C	86	124	8	0.46

class "C" (**Supplementary Tables 6,7**). The degree distribution for these networks followed the typical shape of a power law distribution (**Supplementary Figures 10E,F**).

DISCUSSION

Our reanalysis, and expanded analysis, of previously published data from periodontal disease studies using CoDA techniques improved our ability to detect patterns and correlations in these data and provided new insights into the relationships of organisms, cytokines, and metabolites to the disease process. Analysis of beta-diversity using the clr-transformed datasets detected distinct clustering not observed in the original studies. In the SHT study, unlike the original PCoA analysis that saw no separation of samples by disease class for any "omics" dataset (Califf et al., 2017), we identified clustering of clr-transformed data by both disease class and pocket depth in the NMDS ordination plots, with disease class C and deeper pocket depth in the left cluster and disease class A and shallower pocket depth in the right cluster (**Figure 2H** and **Supplementary Figure 2B**). In the PT study, we did not see clustering of the samples by 16S, cytokine, or metagenomic datasets for any of the classifications (periodontal treatment, overall response, or pocket depth) in the NMDS ordination (**Figure 2**), which concurs with the findings from the original study (Schwarzberg et al., 2014). However, PERMANOVA showed differences between the pre- and posttreatment samples and disease class in the PT and SHT studies' 16S data, findings not determined in the original studies due to the high level of individual variability (**Tables 1–3**).

The original PT study reported significant relationships between the combined abundance of *P. gingivalis*, *Fusobacterium nucleatum*, *T. forsythia*, and *T. denticola*, which are periodontal pathogens, and IL-1β (Vijay Kumar et al., 2018). However, the only significant correlation we observed in the PT study dataset was between IL-1 and *Prevotella*. IL-1 has been associated with periodontal disease severity (Offenbacher et al., 2007), and *Prevotella* includes species in the orange complex associated with periodontal disease (Socransky et al., 1998). For the SHT study, we observed significant bacterial–metabolite correlations among the diseased samples, which the original study did not investigate (**Supplementary Figures 5A,B**). Among the samples in all disease classes, *Paludibacter*, a bacterial genus associated with plaque in healthy patients (Chen et al., 2018), was negatively correlated with two metabolites (**Supplementary Figure 5A**). However, selbal identified this genus as more predictive of worsened samples with high pocket depth (**Table 3** and **Supplementary Figure 8D**), so this genus may not be "health-associated" as previously thought. Other genera negatively correlated with metabolites in the samples of all disease classes were *Desulfovibrio*, *Peptococcus*, and *TG5* (**Supplementary Figure 4A**). *Desulfovibrio* species may stimulate the immune response (Dzierżewicz et al., 2010) and have been observed in periodontal pockets (Loubinoux et al., 2002). *Peptococcus* and *TG5* have been found in the oral microbiomes of periodontitis patients (Kumar et al., 2005; Apatzidou et al., 2017). *Acinetobacter*, which has been previously associated with periodontitis samples (Souto et al., 2014), and *Rubrivivax* were positively correlated with metabolites (**Supplementary Figure 5A**). As *Rubrivivax* is not commonly associated with periodontal disease, this genus may be a new route of study. *Treponema*, a "red complex" species, was correlated with most metabolites in the samples from all disease classes (**Supplementary Figure 5A**) and was correlated with one metabolite among the samples that were only within disease class "A" (**Supplementary Figure 4B**). Also significantly correlated with metabolites in disease class "A" samples were *Olsenella* and *Atopobium* (**Supplementary Figure 5B**). In periodontitis patients, *Olsenella* species have been detected in abundance (Chávez de Paz et al., 2004) and *Atopobium* species have been associated with periodontal disease (Kumar et al., 2005; Apatzidou et al., 2017).

The DIABLO multiomics integration uncovered an additional negative relationship between IL-6/IL-10 and *Treponema* and *Schwartzia* in the PT study and many correlations between *Treponema* and *TG5* with many metabolites in the SHT study (**Figures 3A,B**). *Schwartzia*, *Treponema*, and *TG5* have been associated with the biofilms of periodontal disease patients (Socransky et al., 1998; Camelo-Castillo et al., 2015; Apatzidou et al., 2017). Additionally, *T. denticola* has been shown to degrade IL-1β and IL-6 (Miyamoto et al., 2006), and infection

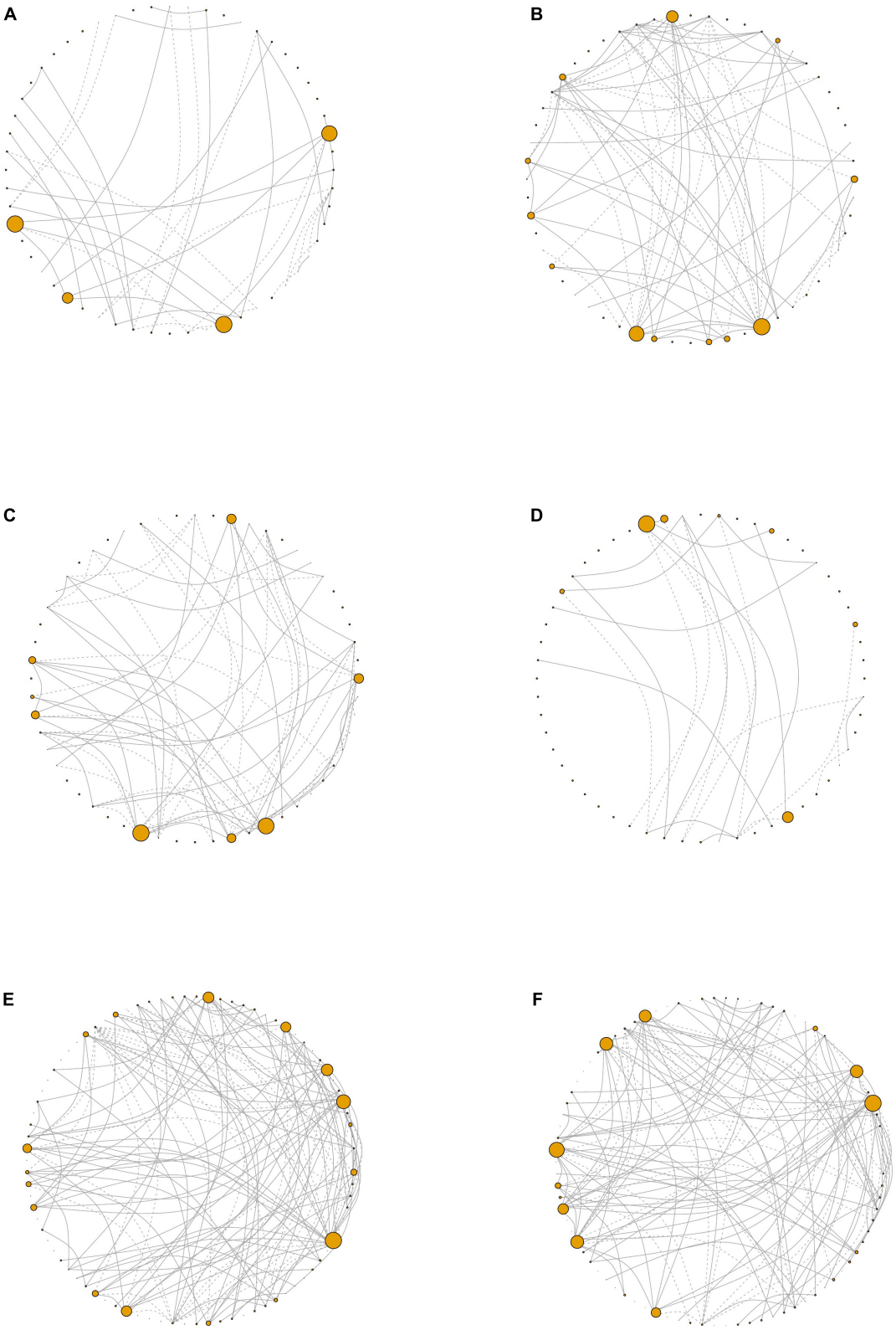

FIGURE 6 | Network visualizations. Network plots derived from the periodontal treatment (PT) study patients whose oral status was designated as **(A)** gingivitis, **(B)** moderate, **(C)** shallow, or **(D)** deep. For the sodium hypochlorite (SHT) study, network plots for biofilms from patients with **(E)** class "A" or **(F)** class "C." Node size corresponds to eigen centrality. *Dashed lines* represent negative correlations and *solid lines* represent positive correlations.

of both *P. gingivalis* and *T. denticola* synergistically stimulated the production of IL-6 by macrophage-like cells (Tamai et al., 2009). Little is known about the association between *Schwartzia*, periodontal disease, and the immune response, so this genus is a potential new target for investigation. For the SHT study multiomics integration, disease class was able to distinguish integrated 16S and metabolomic samples (**Figure 3E**); disease class was also the only variable that distinguished between samples in our NMDS analysis, indicating that this discrimination may be largely due to metabolite differences.

The orange complex described by Socransky et al. (1998) as highly intercorrelated and associated with PD includes species from the genera *Prevotella* and *Fusobacterium*, which were both identified in our selbal analysis of microbial balances for the PT and SHT datasets. *Prevotella* relative abundance predicted pretreatment samples in the PT dataset and *Fusobacterium* relative abundance predicted high pocket depth and posttreatment samples that had worsened (**Table 2** and **Supplementary Figures 6C**, **7D**). *Fusobacterium* was also predictive of disease class "C" in the SHT study (**Table 3** and **Supplementary Figure 9C**). This aligns well with the original PT studies, where *Fusobacterium* was significantly correlated with pocket depth and a decrease in *Prevotella* after treatment was associated with improvement (Schwarzberg et al., 2014; Califf et al., 2017). Some *Desulfobulbus* species likely play a role in the development of periodontal disease (Camelo-Castillo et al., 2015; Cross et al., 2018), and selbal identified this genus as predictive of pretreatment samples, samples that improved, and shallow pocket depth (**Table 2** and **Supplementary Figures 6A,D**, **9D**). Selbal indicated that *Porphyromonas*, the genus that includes a red complex species, was more predictive of deeper pockets in the PT study and disease class "C" in the SHT study (**Tables 2** and **3** and **Supplementary Figures 8C**, **9A**) and was found in the original study as correlating with high pocket depth (Califf et al., 2017). The most commonly identified cytokine by selbal, IL-6, was found in the original study to be significantly associated with severe periodontitis (Delange et al., 2018), while we found that IL-6 levels were predictive of pretreatment samples and samples that improved (**Table 2** and **Supplementary Figures 6B,E**). Additionally, selbal identified CRP as predictive of samples that worsened (**Table 2** and **Supplementary Figure 6E**), which is in contrast to the previous study which did not find a strong association between CRP and periodontal disease status (Delange et al., 2018). CRP was also identified by random forest as one of the two top important cytokines for predicting pocket depth (**Figure 5B**). Random forest also identified *Abiotrophia*, species of which have been isolated from dental plaques (Mikkelsen et al., 2000), as the most stable genus in predicting disease class in the SHT study 16S data, while in the PT study data, *Peptococcus* and *Porphyromonas* were the most important genera in predicting pocket depth (**Figure 5**).

Analysis of correlation networks can provide insights into the complexity, stability, and function of a microbial community (Barberán et al., 2012). The most striking disparity in overall network connectivity occurred in the PT study. The network analysis found that, for the PT study, networks of 16S and cytokines for the samples with deep pockets had fewer edges and lower transitivity (**Table 5**). Fewer inter-nodal connections and a lower overall network connectedness indicate a lack of interdependence of taxa in deeper pockets. Multiple studies have shown that pocket depth is correlated with greater alpha diversity and more pathogenic taxa (Christersson et al., 1992; Stoltenberg et al., 1993; Takeshita et al., 2016). The early stages of periodontal development involve well-known interactions between bacterial species (Nyvad and Kilian, 1987; Diaz et al., 2006; Chalmers et al., 2008), but as biofilms develop and become increasingly anaerobic, more pathogenic species establish within the biofilm (Van Winkelhoff et al., 2002; Vieira Colombo et al., 2016). The fewer connections that we observed in deep pockets could reflect a more random, or less stable, biofilm in the later stages of disease. Additionally, the networks constructed from the samples with shallow pockets had greater transitivity (**Table 5**), which implies the presence of more inter-nodal interactions within the shallow pocket networks and may be indicative of niche filtering, where similarities rather than differences between species allow the persistence of species in an environment (Röttjers and Faust, 2018). Network analysis also revealed that *Aerococcus* had a high eigen centrality value in the networks for disease classes "A" and "C" (**Supplementary Tables 5,6**). Higher eigen centralities indicate that nodes are critical for network stability and may point toward keystone species (Bauer et al., 2010; Mandakovic et al., 2018). While *Aerococcus* species have been found in the biofilms of periodontitis patients (Voropaeva et al., 2008), but as little is known about the association between *Aerococcus* and periodontal disease, this may be an interesting future avenue of study.

We should note potential effects of the study population demographics on our data. Most participants from the PT study had an overweight or obese body mass index, and 37% were smokers, both of which could affect the microbiome composition and inflammation levels measured in this study. Additionally, the PT study participants were American Indian/Alaskan Native, on average over a decade younger, and had a higher prevalence of females (66 *vs*. 44%) than the SHT study participants, so the differences in the results between the two studies could be due to effects of ethnicity, aging, or sex on the oral microbiome and inflammation. Our use of CoDA techniques, which confirmed many of the prior studies' results and uncovered new findings, shows how this approach is a valuable addition to the current methods of microbiome data analysis for investigating oral disease. We have shown how CoDA approaches are especially useful when integrating multiomics due to the scale-invariance that the clr transformation confers on datasets. The identification of CRP as predictive of pocket depth and samples that worsened is a new finding and an important area of further study. We also identified understudied genera potentially important in periodontal disease (*Schwartzia*, *Rubrivivax*, and *Aerococcus*). Furthermore, the ability of unknown metabolites to discriminate between samples in selbal analyses, and the associations we determined between metabolites and particular taxa, highlights the need to study these compounds in the context of periodontal disease.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study and code for all analyses can be found on zenodo: https://doi.org/10.5281/zenodo.4604009 further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by USC Health Sciences Campus Institutional Review Board, the SDSU Institutional Review Board (IRB) and the IRB of the Southern California American Indian Health Clinic. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AO-V and MR performed the computations and generated figures with guidance from LS-H and SK. LS-H wrote the initial draft of the manuscript. SK, AO-V, and MR edited the manuscript. All authors discussed the results and contributed to the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.617949/full#supplementary-material

Supplementary Figure 1 | NMDS ordination plots showing clustering of PT samples by pocket depth. Columns correspond to dataset type; 16S, Cytokine, and Metagenomic datasets are columns one, two, and three, respectively (n = 60, 104, and 22).

Supplementary Figure 2 | NMDS ordination plots showing clustering of SHT samples by pocket depth. Columns correspond to dataset type; 16S, Metabolites, and Metagenomic datasets are columns one, two, and three, respectively (n = 209, 153, and 24).

Supplementary Figure 3 | Beta dispersion analysis by periodontal treatment, response to treatment, and disease class. For the PT study, distance from the centroid of beta dispersion is shown for samples pre- and post-treatment for **(A)** 16S bacterial, **(B)** metabolite, and **(C)** metagenomic datasets and for response to treatment for **(D)** 16S bacterial, **(E)** metabolite, and **(F)** metagenomic datasets. Larger distances indicate samples which are far from the group centroid, small distances indicate samples which are close to the group centroid. For the SHT study, distance from the centroid is shown for samples by disease class for **(G)** 16S bacterial, **(H)** metabolite, and **(I)** metagenomic datasets.

Supplementary Figure 4 | Bacterial-cytokine correlation matrix for post-treatment, worsened samples. All spaces with color are p-adj <0.05. Blue indicates a positive correlation; Dark orange indicates a negative correlation.

Supplementary Figure 5 | Correlation matrices between comparisons of different SHT datasets (bacterial 16S-metabolite and bacterial 16S-metagenomic). **(A)** Bacterial-metabolite correlations on a combined dataset from all disease classes; **(B)** Bacterial-metabolite correlations on disease class "A." All spaces with color are p-adj <0.05. Blue indicates a positive correlation, Dark orange indicates a negative correlation.

Supplementary Figure 6 | Microbial and cytokine balances for PT dataset at different conditions computed with selbal, where numerator genera are more relatively abundant than denominator genera for higher balance values. Balances are shown for samples before and after periodontal treatment for **(A)** bacterial 16S data, **(B)** cytokine, and **(C)** metagenomic data. Balances are shown for improved and worsened samples of **(D)** bacteria 16S data, **(E)** cytokine, and **(F)** metagenomic data.

Supplementary Figure 7 | Microbial and cytokine balances for PT dataset for improved **(A–C)** and worsened **(D–F)** samples computed with selbal. Balances before and after treatment are shown for samples that improved for **(A)** bacterial 16S data **(B)** cytokine, and **(C)** metagenomic data. Balances before and after treatment are shown for samples that worsened for **(D)** bacterial 16S data, **(E)** cytokine, and **(F)** metagenomic data.

Supplementary Figure 8 | Selbal-computed microbial and cytokine balances for PT dataset associated with the sum of all pocket depths. Balances for improved samples by pocket depth (response variable) are shown for **(A)** 16S bacterial, **(B)** cytokine, and **(C)** metagenomic datasets. Balances for worsened samples by pocket depth are shown for **(D)** 16S bacterial, **(E)** cytokine, and **(F)** metagenomic datasets.

Supplementary Figure 9 | Selbal-computed balances for SHT dataset for microbial and metabolite balances by different characteristics. For samples with disease class either "A" or "C," balances shown are for **(A)** 16S bacterial, **(B)** metabolite, and **(C)** metagenomic data. Balances for samples with disease class "A" associated with pocket depth sum are shown for **(D)** 16S bacterial, **(E)** metabolite, and **(F)** metagenomic datasets. Balances for disease class "C" samples associated with pocket depth sum are shown for **(G)** 16S bacterial, **(H)** metabolite, and **(I)** metagenomic datasets.

Supplementary Figure 10 | Network degree distributions. For the PT study for networks with OTUs and cytokines, degree distribution of inferred network from patients who had **(A)** gingivitis or **(B)** moderate disease, **(C)**, shallow or **(D)** deep pockets. For the SHT study, degree distribution of inferred network from patients who had class (E) "A" or (F) "C" disease.

REFERENCES

Aitchison, J. (1982). The statistical analysis of compositional data. *J. R. Stat. Soc. B* 44, 139–160.

Apatzidou, D., Lappin, D. F., Hamilton, G., Papadopoulos, C. A., Konstantinidis, A., and Riggio, M. P. (2017). Microbiome associated with peri-implantitis versus periodontal health in individuals with a history of periodontal disease. *J. Oral Microbiol.* 9:1325218. doi: 10.1080/20002297.2017.1325218

Barberán, A., Bates, S. T., Casamayor, E. O., and Fierer, N. (2012). Using network analysis to explore co-occurrence patterns in soil microbial communities. *ISME J.* 6, 343–351. doi: 10.1038/ismej.2011.119

Bauer, B., Jordán, F., and Podani, J. (2010). Node centrality indices in food webs: rank orders versus distributions. *Ecol. Complexity* 7, 471–477. doi: 10.1016/j.ecocom.2009.11.006

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857.

Califf, K. J., Schwarzberg-Lipson, K., Garg, N., Gibbons, S. M., Caporaso, J. G., Slots, J., et al. (2017). Multi-omics analysis of periodontal pocket microbial communities pre- and posttreatment. *mSystems* 2:e00016-17. doi: 10.1128/mSystems.00016-17

Camelo-Castillo, A. J., Mira, A., Pico, A., Nibali, L., Henderson, B., Donos, N., et al. (2015). Subgingival microbiota in health compared to periodontitis and the influence of smoking. *Front. Microbiol.* 6:119. doi: 10.3389/fmicb.2015.00119

Chalmers, N. I., Palmer, R. J., Cisar, J. O., and Kolenbrander, P. E. (2008). Characterization of a *Streptococcus* sp. *Veillonella* sp. community micromanipulated from dental Plaque. *J. Bacteriol.* 190, 8145–8154. doi: 10.1128/jb.00983-08

Chávez de Paz, L. E., Molander, A., and Dahlén, G. (2004). Gram-positive rods prevailing in teeth with apical periodontitis undergoing root canal treatment. *Int. Endodontic J.* 37, 579–587. doi: 10.1111/j.1365-2591.2004.00845.x

Chen, C., Hemme, C., Beleno, J., Shi, Z. J., Ning, D., Qin, Y., et al. (2018). Oral microbiota of periodontal health and disease and their changes after nonsurgical periodontal therapy. *ISME J.* 12, 1210–1224. doi: 10.1038/s41396-017-0037-1

Christersson, L. A., Fransson, C. L., Dunford, R. G., and Zambon, J. J. (1992). Subgingival distribution of periodontal pathogenic microorganisms in adult periodontitis. *J. Periodontol.* 63, 418–425. doi: 10.1902/jop.1992.63.5.418

Cross, K. L., Chirania, P., Xiong, W., Beall, C. J., Elkins, J. G., Giannone, R. J., et al. (2018). Insights into the evolution of host association through the isolation and characterization of a novel human periodontal pathobiont, *Desulfobulbus oralis*. *mBio* 9:e02061-17.

Csardi, G., and Nepusz, T. (2006). *The iGraph Software Package for Complex Network Research: R package. InterJournal*.

Delange, N., Lindsay, S., Lemus, H., Finlayson, T. L., Kelley, S. T., and Gottlieb, R. A. (2018). Periodontal disease and its connection to systemic biomarkers of cardiovascular disease in young American Indian/Alaskan natives. *J. Periodontol.* 89, 219–227. doi: 10.1002/jper.17-0319

Diaz, P. I., Chalmers, N. I., Rickard, A. H., Kong, C., Milburn, C. L., Palmer, R. J. Jr., et al. (2006). Molecular characterization of subject-specific oral microflora during initial colonization of enamel. *Appl. Environ. Microbiol.* 72, 2837–2848. doi: 10.1128/aem.72.4.2837-2848.2006

Dzierżewicz, Z., Szczerba, J., Lodowska, J., Wolny, D., Gruchlik, A., Orchel, A., et al. (2010). The role of Desulfovibrio desulfuricans lipopolysaccharides in modulation of periodontal inflammation through stimulation of human gingival fibroblasts. *Arch. Oral Biol.* 55, 515–522. doi: 10.1016/j.archoralbio.2010.05.001

Eke, P. I., Dye, B. A., Wei, L., Thornton-Evans, G. O., and Genco, R. J. (2012). Prevalence of periodontitis in adults in the United States: 2009 and 2010. *J. Dent. Res.* 91, 914–920. doi: 10.1177/0022034512457373

Faveri, M., Mayer, M. P. A., Feres, M., De Figueiredo, L. C., Dewhirst, F. E., and Paster, B. J. (2008). Microbiological diversity of generalized aggressive periodontitis by 16S rRNA clonal analysis. *Oral Microbiol. Immunol.* 23, 112–118. doi: 10.1111/j.1399-302x.2007.00397.x

Gloor, G. B., Macklaim, J. M., Pawlowsky-Glahn, V., and Egozcue, J. J. (2017). Microbiome datasets are compositional: and this is not optional. *Front. Microbiol.* 8:2224. doi: 10.3389/fmicb.2017.02224

Griffen, A. L., Beall, C. J., Campbell, J. H., Firestone, N. D., Kumar, P. S., Yang, Z. K., et al. (2012). Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME J.* 6, 1176–1185. doi: 10.1038/ismej.2011.191

Kroes, I., Lepp, P. W., and Relman, D. A. (1999). Bacterial diversity within the human subgingival crevice. *Proc. Natl. Acad. Sci. U.S.A.* 96, 14547–14552. doi: 10.1073/pnas.96.25.14547

Kumar, P. S., Griffen, A. L., Moeschberger, M. L., and Leys, E. J. (2005). Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. *J. Clin. Microbiol.* 43, 3944–3955. doi: 10.1128/jcm.43.8.3944-3955.2005

Kumar, P. S., Leys, E. J., Bryk, J. M., Martinez, F. J., Moeschberger, M. L., and Griffen, A. L. (2006). Changes in periodontal health status are associated with bacterial community shifts as assessed by quantitative 16S cloning and sequencing. *J. Clin. Microbiol.* 44, 3665–3673. doi: 10.1128/jcm.00317-06

Kurtz, Z. D., Müller, C. L., Miraldi, E. R., Littman, D. R., Blaser, M. J., and Bonneau, R. A. (2015). Sparse and compositionally robust inference of microbial ecological networks. *PLoS Comput. Biol.* 11:e1004226. doi: 10.1371/journal.pcbi.1004226

Lamont, R. J., and Jenkinson, H. F. (1998). Life below the gum line: pathogenic mechanisms of Porphyromonas gingivalis. *Microbiol. Mol. Biol. Rev.* 62, 1244–1263. doi: 10.1128/mmbr.62.4.1244-1263.1998

Loubinoux, J., Bisson-Boutelliez, C., Miller, N., and Le Faou, A. E. (2002). Isolation of the provisionally named Desulfovibrio fairfieldensis from human periodontal pockets. *Oral Microbiol. Immunol.* 17, 321–323. doi: 10.1034/j.1399-302x.2002.170510.x

Mandakovic, D., Rojas, C., Maldonado, J., Latorre, M., Travisany, D., Delage, E., et al. (2018). Structure and co-occurrence patterns in microbial communities under acute environmental stress reveal ecological factors fostering resilience. *Sci. Rep.* 8:5875.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., et al. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6, 610–618. doi: 10.1038/ismej.2011.139

Mikkelsen, L., Theilade, E., and Poulsen, K. (2000). Abiotrophia species in early dental plaque. *Oral Microbiol. Immunol.* 15, 263–268. doi: 10.1034/j.1399-302x.2000.150409.x

Miyamoto, M., Ishihara, K., and Okuda, K. (2006). The *Treponema denticola* surface protease dentilisin degrades interleukin-1β (IL-1β), IL-6, and tumor necrosis factor alpha. *Infect. Immun.* 74, 2462–2467. doi: 10.1128/iai.74.4.2462-2467.2006

Nyvad, B., and Kilian, M. (1987). Microbiology of the early colonization of human enamel and root surfaces in vivo. *Scand. J. Dent. Res.* 95, 369–380. doi: 10.1111/j.1600-0722.1987.tb01627.x

Offenbacher, S., Barros, S. P., Singer, R. E., Moss, K., Williams, R. C., and Beck, J. D. (2007). Periodontal disease at the biofilm–gingival interface. *J. Periodontol.* 78, 1911–1925. doi: 10.1902/jop.2007.060465

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., et al. (2019). *vegan: Community Ecology Package: R package version 2.5-6*.

Palarea-Albaladejo, J., and Martín-Fernández, J. A. (2015). zCompositions — R package for multivariate imputation of left-censored data under a compositional approach. *Chemomet. Intell. Lab. Syst.* 143, 85–96. doi: 10.1016/j.chemolab.2015.02.019

Paster, B. J., Boches, S. K., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., et al. (2001). Bacterial diversity in human subgingival plaque. *J. Bacteriol.* 183, 3770–3783. doi: 10.1128/jb.183.12.3770-3783.2001

Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., et al. (2012). Scikit-learn: machine learning in python. *arXiv* [Preprint] arXiv:1201.0490v4.

Quinn, T. P., and Erb, I. (2019). Another look at microbe–metabolite interactions: how scale invariant correlations can outperform a neural network. *bioRxiv* [Preprint] doi: 10.1101/847475

Quinn, T. P., Erb, I., Richardson, M. F., and Crowley, T. M. (2018). Understanding sequencing data as compositions: an outlook and review. *Bioinformatics* 34, 2870–2878. doi: 10.1093/bioinformatics/bty175

Revelle, W. (2020). *psych: Procedures for Psychological, Psychometric, and Personality Research: R package*. Evanston, IL: Northwestern University.

Rivera-Pinto, J., Egozcue, J. J., Pawlowsky-Glahn, V., Paredes, R., Noguera-Julian, M., and Calle, M. L. (2018). Balances: a new perspective for microbiome analysis. *mSystems* 3:e00053-18.

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2584. doi: 10.7717/peerj.2584

Rohart, F., Gautier, B., Singh, A., and Lê Cao, K.-A. (2017). mixOmics: an R package for 'omics feature selection and multiple data integration. *PLoS Comput. Biol.* 13:e1005752. doi: 10.1371/journal.pcbi.1005752

Röttjers, L., and Faust, K. (2018). From hairballs to hypotheses–biological insights from microbial networks. *FEMS Microbiol. Rev.* 42, 761–780. doi: 10.1093/femsre/fuy030

Schwarzberg, K., Le, R., Bharti, B., Lindsay, S., Casaburi, G., Salvatore, F., et al. (2014). The personal human oral microbiome obscures the effects of treatment on periodontal disease. *PLoS One* 9:e86708. doi: 10.1371/journal.pone.0086708

Sisk-Hackworth, L., and Kelley, S. T. (2020). An application of compositional data analysis to multiomic time-series data. *Nucleic Acids Res. Genomics Bioinform.* 2:lqaa079.

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., and Kent, R. L. Jr. (1998). Microbial complexes in subgingival plaque. *J. Clin. Periodontol.* 25, 134–144. doi: 10.1111/j.1600-051x.1998.tb02419.x

Souto, R., Silva-Boghossian, C. M., and Colombo, A. P. (2014). Prevalence of *Pseudomonas aeruginosa* and *Acinetobacter* spp. in subgingival biofilm and saliva of subjects with chronic periodontal infection. *Braz. J. Microbiol.* 45, 495–501. doi: 10.1590/s1517-83822014000200017

Stoltenberg, J. L., Osborn, J. B., Pihlstrom, B. L., Herzberg, M. C., Aeppli, D. M., Wolff, L. F., et al. (1993). Association between cigarette smoking, bacterial

pathogens, and periodontal status. *J. Periodontol.* 64, 1225–1230. doi: 10.1902/jop.1993.64.12.1225

Takeshita, T., Kageyama, S., Furuta, M., Tsuboi, H., Takeuchi, K., Shibata, Y., et al. (2016). Bacterial diversity in saliva and oral health-related conditions: the Hisayama Study. *Sci. Rep.* 6:22164.

Tamai, R., Deng, X., and Kiyoura, Y. (2009). *Porphyromonas gingivalis* with either *Tannerella forsythia* or *Treponema denticola* induces synergistic IL-6 production by murine macrophage-like J774.1 cells. *Anaerobe* 15, 87–90. doi: 10.1016/j.anaerobe.2008.12.004

Torres, P. J., Thompson, J., Mclean, J. S., Kelley, S. T., and Edlund, A. (2019). Discovery of a novel periodontal disease-associated bacterium. *Microb. Ecol.* 77, 267–276. doi: 10.1007/s00248-018-1200-6

Van Winkelhoff, A. J., Loos, B. G., Van Der Reijden, W. A., and Van Der Velden, U. (2002). *Porphyromonas gingivalis*, *Bacteroides forsythus* and other putative periodontal pathogens in subjects with and without periodontal destruction. *J. Clin. Periodontol.* 29, 1023–1028. doi: 10.1034/j.1600-051x.2002.291107.x

Vieira Colombo, A. P., Magalhães, C. B., Hartenbach, F. A. R. R., Martins Do Souto, R., and Maciel Da Silva-Boghossian, C. (2016). Periodontal-disease-associated biofilm: a reservoir for pathogens of medical importance. *Microb. Pathog.* 94, 27–34. doi: 10.1016/j.micpath.2015.09.009

Vijay Kumar, P. K., Gottlieb, R. A., Lindsay, S., Delange, N., Penn, T. E., Calac, D., et al. (2018). Metagenomic analysis uncovers strong relationship between periodontal pathogens and vascular dysfunction in American Indian population. *bioRxiv* [Preprint] doi: 10.1101/250324

Voropaeva, E. A., Bairakova, A. L., Bichucher, A. M., D'iakov, V. L., and Kozlov, L. V. (2008). [Protease activity of microflora in the oral cavity of patients with periodontitis]. *Biomeditsinskaia khimiia* 54, 706–711.

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/aem.00062-07

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag New York.

Ximénez-Fyvie, L. A., Haffajee, A. D., and Socransky, S. S. (2000). Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. *J. Clin. Periodontol.* 27, 648–657. doi: 10.1034/j.1600-051x.2000.027009648.x

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The Oxidative Response of Human Monocytes to Surface Modified Commercially Pure Titanium

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Cellular responses to implanted biomaterials are key to understanding osseointegration. The aim of this investigation was to determine the *in vitro* priming and activation of the respiratory burst activity of monocytes in response to surface-modified titanium. Human peripheral blood monocytes of healthy blood donors were separated, then incubated with surface-modified grade 2 commercially pure titanium (CPT) disks with a range of known surface energies and surface roughness for 30- or 60-min. Secondary stimulation by phorbol 12-myrisate 13-acetate (PMA) following the priming phase, and luminol-enhanced-chemiluminescence (LCL) was used to monitor oxygen-dependent activity. Comparison among groups was made by incubation time using one-way ANOVA. One sample from each group for each phase of the experiment was viewed under scanning electron microscopy (SEM) and qualitative comparisons made. The results indicate that titanium is capable of priming peripheral blood monocytes following 60-min incubation. In contrast, 30 min incubation time lead to reduced LCL on secondary stimulation as compared to cells alone. At both time intervals, the disk with the lowest surface energy produced significantly less LCL compared to other samples. SEM examination revealed differences in surface morphology at different time points but not between differently surface-modified disks. These results are consistent with the hypothesis that the titanium surface characteristics influenced the monocyte activity, which may be important in regulating the healing response to these materials.

Keywords: titanium, monocytes, surface roughness, wettability, surface energy, chemiluminescence, oxidative response, dental implants

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INTRODUCTION

The use of endosseous dental implants to support restorations replacing missing teeth has become well established since the introduction of titanium dental implants (1, 2). The successful incorporation and rigid fixation of an implant within the surrounding bone was defined as osseointegration (3). While implants with surface characteristics that allow osseointegration have been available for many years, the exact surface characteristics necessary for optimal

osseointegration remain to be completely elucidated, although it is known that a key feature is the highly stable passivating layer of titanium oxide that covers the titanium surface (4). It is also thought that the combined effects of surface energy, chemistry, and topography may play a major role during the initial phases of the biological response to the implant (5–7).

The effect of surface roughness, in particular, has been evaluated in multiple investigations. In *vivo* studies have demonstrated that increasing surface roughness of an implant results in an increase in bone to implant contact (8, 9). The vascular nature of bone and the inevitable surgical trauma created by the implant site preparation, ensures that the first tissue to come into contact with an endosseous implant is blood with its complement of inflammatory cells (10–12). While these early interactions between the inflammatory cells and the implant surface are thought to be important, much still needs to be determined about the nature of these interactions.

The cells of the mononuclear phagocyte system play a crucial role in the regulation of chronic inflammation and wound healing. Monocytes are also thought to have a significant role in the regulation of osseous metabolism, both in bone resorption and bone formation (13). Monocyte migration and spreading is influenced by the surface energy and roughness of the material on which the cells are attached (14, 15). It has been shown that macrophage attachment to surfaces increases with increasing surface roughness (16). More recently, it was found that the number of monocytes attached to blasted titanium surfaces was significantly greater than to machined titanium surfaces (17). These observations suggest that macrophage adherence may provide signals that induce specific macrophage functions (18). There is concern, however, that attachment of monocytes/macrophages to implant surfaces *in vivo* may jeopardize successful osseointegration since these cells are capable of inducing bone resorption and chronic inflammation.

Initially, the monocyte is primed by a low-grade stimulus, which elevates the cell to a heightened but subthreshold level of activation. Once activated, primed monocytes undergo respiratory burst activity and generate enhanced levels of reactive oxygen species and have higher levels of degranulation and greater phagocytic activity when compared to resting state monocytes. The respiratory burst, through the generation of reactive oxygen species, produces chemiluminescence (CL). The level of activity can be measured in a chemiluminometer. It is possible that the level of monocyte priming, and activation may be directly linked to the rate of healing following implant placement and long-term stability of the rigid bone-implant interface, although this has not been verified. Since the attachment of cells to a titanium surface is an important phenomenon in the area of clinical implant dentistry, a major consideration in designing implants has been to produce surfaces that promote desirable responses in the cells and tissues. Following machining, the surface roughness of an implant may be altered by mechanical methods such as mechanical polishing and sandblasting or by chemical methods, which include anodizing, etching and coating. These processes can change the surface properties of the commercially pure titanium (CPT). In particular, surface roughness and surface energy or wettability, measured as contact angle, have been shown to be affected by various surface conditioning treatments of grade 2 CPT (19). These fine features are significant in promoting osteoblast adherence, bone formation and attachment to the implant surface (20, 21).

The purpose of this *in vitro* study was to determine the priming and activation levels of the respiratory burst activity of human peripheral blood monocytes in response to surface-modified grade 2 CPT disks with a range of surface energies and surface roughness. Secondly, to envisage by scanning electron microscopy (SEM), the surface characteristics of these human peripheral blood monocytes adherent to the surface-modified CPT. It was hypothesized that human peripheral blood monocytes have a greater priming and respiratory burst activation response to CPT of higher surface energy than to those of lower surface energy; and secondly, that human peripheral blood monocytes have a greater priming and respiratory burst activation response to CPT of greater roughness than to those of lesser roughness. We postulate that the level of monocyte priming, and activation may be linked to the rate and type of healing following implant placement and the long-term stability of the rigid bone-implant interface. It is thought that a specific range of monocyte priming and activation levels may be conducive to a healing rate that allows the bone to be properly organized and mineralized allowing the development of a rigid bone-implant interface that will be stable under loading for a long period of time and further, that surface energy and surface roughness of the biomaterial will determine the level of priming and respiratory burst activity of the adherent monocytes.

MATERIAL AND METHODS

Isolation of Human Blood Monocytes

Whole blood was collected from healthy adult donors at the Central Indiana Regional Blood Center in Indianapolis (CIRBC), Indiana, and purchased under an IRB-approved contract. No distinctions were made between race, age or sex. Health is routinely determined retrospectively by testing for standard infectious diseases according to a standard CRIBC protocol. Blood (1 unit or 470 ml/experiment) was collected in citrate phosphate dextrose solution anticoagulant bags and centrifuged at 2000x g at 4°C for 4 min. Buffy coat layers were drawn off by the blood center and were used as the source of monocytes. Once in the laboratory, the buffy coat was diluted in a 1:1 ratio with RPMI (Sigma Chemicals; St. Louis, MO) to maximize efficiency of separation. Subsequently the monocytes were isolated from the buffy layer by a variation of the double dextran method (22–25). This involved placing 3 ml of HISTOPAQUE-1119 (Sigma Chemicals), a density gradient cell separation medium, in 15 ml conical test tubes at room temperature. Next, 3 ml of HISTOPAQUE-1077 (Sigma Chemicals) was carefully layered on with a pipette, and 6 ml of buffy coat/RPMI mixture was layered on top very carefully, so as to not disrupt the histopaque layers. The buffy coats and separation medium were then centrifuged at 1700 rpm for 35 min at room temperature (18-26°C). Following centrifugation, the plasma was on top of the mononuclear layer that was above a cloudy layer containing

clumped cells and separating medium, which was above the granulocyte layer. The layer containing granulocytes sits directly on top of the heavy Red Blood Cell (RBC) layer. This lower layer is a pellet of RBC. Following centrifugation, the monocyte layer was drawn off with a bulb pipette and washed twice with 10 ml of Phosphate Buffered Saline (PBS), then centrifuged at 950 rpm for 10 min, and the supernatant discarded. This wash was then repeated with 10 ml RPMI. The cells were then resuspended in 10 ml RPMI. The cell suspension was placed in a polystyrene culture dish, covered and incubated at 37°C for at least 1 h. The culture dish was then gently shaken and the non-adherent cells (lymphocytes) were removed by pipetting and rinsing with warm (37°C) RPMI medium. The monocytes were then rinsed with a Calcium and Magnesium-free Hanks Balanced Salt solution (Sigma Chemicals) and detached by scrapping the cells off the petri dish with a sterile cell-lifter instrument (Fisher Scientific; Itasca IL). The cells were then pelletized by centrifugation at 950 rpm for 10 min at 4°C, and resuspended in 10 ml RPMI, stained with Trypan blue (Sigma Chemicals) and counted with a hemocytometer to determine the number of monocytes and their viability. The cells were then resuspended to a final concentration of 1.0 X 10^6 cells/ml.

Preparation of Specimens

Rods of CPT ASTM grade 2 were cut into 120 (5 x 5 x 1 mm) disks and prepared in similar fashion to the methods described by Lim (19). The specimens were polished using #800-grit SiC metallographic papers on all sides until a visually uniform surface was obtained. All specimens were washed in distilled water and cleaned in an ultrasonic bath and dried at room temperature. Each disk was then randomly assigned to one of six treatment groups, including the control, until there were 20 specimens in each group (**Table 1**). The particular surface modifications were chosen to reflect a range of surface roughness and wettability (**Table 2**). The six processes were divided into four treatment types: mechanical (control) to stimulate a machined surface, chemical (groups 4 and 5), mechano-chemical (group 2) and oxidation (groups 3 and 6). Following preparation, the samples were sterilized by gamma radiation (Wright Medical Technology; Arlington, TN) prior to incubation with the monocytes.

Development of Methodology

After a series of pilot experiments, it was found that PMA 1 x 10^{-5} M with luminol 1 x 10^{-6} M provided the optimal conditions for a maximal cell response in our system (**Figure 1**).

Chemiluminescence Assay 60-Minutes Incubation

The monocytes obtained from the buffy coat separation were stored at 4°C until used. 500 µl of monocyte suspension (1 x 10^6

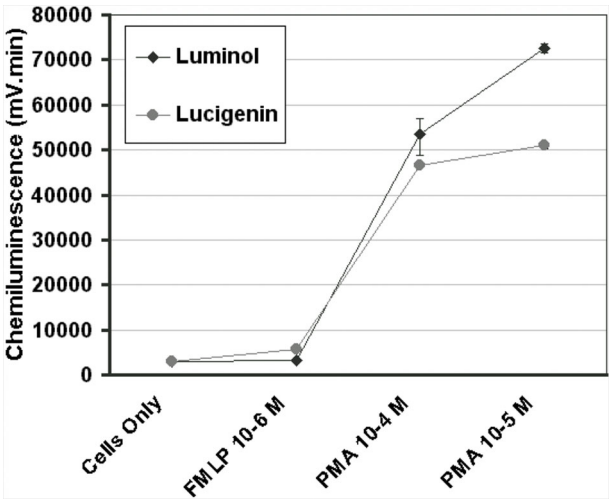

FIGURE 1 | Concentration curves for PMA and FMLP in the luminescence system, as indicated by the total chemiluminescence response of monocytes activated with PMA 10^{-4} M, PMA 10^{-5} M, or FMLP 10^{-6} M with luminol 10^{-6} M or lucigenin 10^{-4} M. (n=3) (Error bars=% covariance).

TABLE 1 | List of surface modification conditions of commercially pure titanium disks.

Titanium Group	Surface modification
(1) Mechanical treatment group (control)	
1	As-polished with #800 grit SiC metallographic paper.
(2) Mechano-chemical group	
2	Sand-blasting (50 µm alumina particles) at 120 psi for 1 minute with a fixed distance (1 cm) between the sample of the surface and blasting tip, followed by chemical treatment in boiling $HCl/H_2SO_4/H_2O$ (20ml/20ml/260ml) for 3 hours.
(3) Oxidation treatment group	
3	In 70°C 5 M NaOH for 24 hours, followed by in-air oxidation at 600°C for 1 hour.
6	$HF/HNO_3/H_2O$ (1/1/2 by fraction) for 10 seconds, followed by process (3).
(4) Chemical treatment group	
4	Immersed in boiling 3% H_2O_2 for 6 hours.
5	Boiling in 5% H_2SO_4 for 15 hours.

All samples (all sides) prior to the treatments were mechanically polished under the same condition as control samples (group 1).

TABLE 2 | Relationship between surface roughness and contact angle on grade 2 commercially pure titanium using distilled water.

Titanium Group	R_a (μm) (Std Dev)	R_{max} (μm) (Std Dev)	Contact angle Water (°) (Std Dev)
1	0.57 (± 0.25)	3.41 (± 2.05)	60.19 (± 5.06)
2	1.69 (± 0.99)	9.73 (± 5.85)	58.98 (± 1.69)
3	1.52 (± 1.21)	7.52 (± 6.39)	16.88 (± 1.67)
4	0.61 (± 0.28)	3.73 (± 1.19)	58.48 (± 1.61)
5	2.15 (± 0.75)	14.14 (± 5.94)	72.99 (± 2.40)
6	2.38 (± 0.12)	14.53 (± 0.93)	10.51 (± 1.25)

**All data from Lim (Lim YJ 2000).*
R_a, Average roughness.
R_{max}, Maximum roughness.

TABLE 3 | Protocol design for chemiluminescence assay with 30-minutes priming.

Variables	Priming Phase	Activation Phase	Total CL
	Measurement Period 0-30 min ↓ 10^{-6} Luminol	**Measurement Period 30-120 min ↓ 10^{-5} PMA**	**Measurement Period 0-120 min**
Blank	1 cuvette/replicate →		
Cells Only	3 reps →		
CPT group 1	3 reps →		
CPT group 2	3 reps →		
CPT group 3	3 reps →		
CPT group 4	3 reps →		
CPT group 5	3 reps →		
CPT group 6	3 reps → 22 cuvettes/ experiment		

monocytes), 100 μl luminol 10^{-6} M and 300 μl of phosphate buffered saline (PBS) were dispensed into each reaction cuvette. One experimental or control disk was placed into each reaction cuvette with the exception of the monocyte control cuvettes and the blank control cuvette. The monocytes were incubated with the disks for 60-min at 37°C. Four repeats of each treated disk were used in each of two experimental runs giving an n of 8. There was also one cell only control in each run. The reaction was followed in a Model 1251 BioOrbit Luminometer (Turku, Finland) for 1 h representing the priming phase. After 1 h the luminometer was programmed to dispense PMA 10^{-5} M to activate the cells as a secondary stimulant. The reaction was followed for an additional 1 h representing the activation phase. Chemiluminescent output was measured in millivolts (mV) and data captured using the Multiuse Data Handling Program (BioOrbit, Turku, Finland). The integral or total mV output versus time of the monocytes was calculated and reported in mV*min.

Chemiluminescence Assay 30-Minutes Incubation

The cuvettes containing the CPT disks were prepared in a similar manner to the first assay but in triplicates, with two runs giving an n of 6. This allowed space for a triplicate of cell only controls and one control cuvette containing only the reagents in each run. In this experiment the monocytes were incubated with the disks for 30-min at 37°C. After 30-min, the luminometer was programmed to dispense PMA 10^{-5} M to activate the cells as a secondary stimulant. The reaction was followed for an additional 90-min representing the activation phase (**Table 3**).

Evaluation by Scanning Electron Microscopy

One sample for each of the conditioned CPT groups with the incubated monocytes was fixed in 3% gluteraldehyde after 30-min and 60-min incubation; immediately after activation with PMA 10^{-5} M following 60-min incubation; and 60-min following activation with PMA 10^{-5} M following 30-min incubation. They were then post-fixed in osmium tetroxide, dehydrated through a graded series of ethanol, chemically dried in HMSD, attached to aluminium with epoxy resin, sputter coated with 60/40 gold/palladium alloy, and examined at 25 kV by SEM. A representative scanning electron photomicrograph was taken at a magnification of 1250x and 5000x.

Statistical Methods

The data was summarized based on different categories of titanium disks and incubation time. Group mean and standard deviation within each of the categories was calculated. Before making the comparison between the different titanium groups, a regression model was fit to test for the significant effects of titanium group, incubation time and their potential interaction. The interaction between treatment group and incubation time was significant based on the regression model ($p < 0.0001$), so multiple comparisons between groups by incubation time were performed. These comparisons among the groups were made using one-way ANOVA models by incubation time, with Tukey's multiple range test, adjusted for the control level.

RESULTS

The monocyte LCL following 60-min incubation with the titanium samples and following 60-min activation with PMA are given in **Table 4** and for 30-min incubation and 90-min activation in **Table 5**. LCL above background levels was not detected until stimulation by PMA had occurred, indicating that the titanium surface alone did not stimulate the cells. Thus, the priming and activation phase were considered together in determining the total LCL produced. In order to adjust for the control level and reduce the change in variance between each treatment group, a proportional change from control was

TABLE 4 | Monocyte CL for run 1 and run 2. PMA 10^{-5} M, luminol 10^{-6} M, 60-minutes priming and 60-minutes post-activation.

CPT Group	Run 1		Run 2	
	Mean monocyte CL (mV.min x 10^5)	% Covariance	Mean monocyte CL (mV.min x 10^5)	% Covariance
Cells Only	0.38		0.17	
1	1.58	7.93	1.09	3.78
2	1.53	2.59	1.10	4.45
3	1.45	3.60	1.05	5.46
4	1.36	2.99	0.97	14.19
5	1.18	9.71	0.82	5.34
6	1.30	1.10	1.03	3.32

calculated by subtracting control value from observed value and divided by the control value. All analyses were based on these 'adjusted' LCL values. Further, two potential outlier samples were excluded from the analysis of the data presented.

Table 6 provides a summary of the proportional change of monocyte LCL value, categorized by six different treatment groups for each of the incubation times. The patterns of the percentage change of monocyte LCL values among treatment groups were similar in each of the combined experimental runs except for the changing direction. The negative value for the 30-min incubation time indicates that the mean of the monocyte LCL was smaller than the mean of the control, while the positive value for 60-min incubation indicates that the mean of the monocyte LCL was larger than the mean of the control. The means of monocyte CL for 60-min incubation was significantly higher than zero (p-value was 0.0010, 0.0011, 0.0020, 0.0027, 0.0184 and 0.0035 for treatment groups 1, 2, 3, 4, 5, and 6, respectively), which means that the mean of LCL value in each treatment group was significantly higher than the cell only control group. However, at 30-min incubation the means of monocyte LCL values was significantly lower than zero ($p < 0.0001$ for each group), which means the mean of LCL value in each treatment group was significantly lower than the cell only control group.

The proportional change of monocyte LCL value always had the least response in group 5, regardless of experimental run or incubation time. **Figure 2** reveals that priming of monocytes incubated with group 5 at both incubation times was significantly less than the other groups ($p < 0.001$). No significant differences between other groups were detected.

TABLE 5 | Monocyte CL for run 3 and run 4. PMA 10^{-5} M, luminol 10^{-6} M, 30-minutes priming and 90-minutes post-activation.

CPT Group	Run 3		Run 4	
	Mean monocyte CL (mV.min x 10^5)	% Covariance	Mean monocyte CL (mV.min x 10^5)	% Covariance
Blank Control	0.044		0.042	
Cells Only	2.84		0.382	1.061
1	2.43	6.53	0.326	25.874
2	2.29	3.85	0.326	1.998
3	2.34	4.68	0.322	2.234
4	2.45	3.47	0.321	5.220
5	1.51	6.21	0.229	4.770
6	2.41	0.90	0.329	1.514

TABLE 6 | Summaries of proportional change of monocyte chemiluminescence by CPT groups, under 60- or 30-minutes incubation.

CPT Group	60-minutes Incubation Mean (Std. Dev)	30-minutes Incubation Mean (Std. Dev)
1	4.29 (1.30)	-0.16 (0.05)
2	4.26 (1.36)	-0.17 (0.04)
3	4.01 (1.34)	-0.17 (0.04)
4	3.70 (1.44)	-0.15 (0.04)
5	2.98 (1.00)*	-0.43 (0.05)*
6	3.76 (1.47)	-0.15 (0.01)

**significant at P <0.05.*

Scanning Electron Microscopy

SEM evaluation of the surface-modified CPT disks revealed differences in surface topography between treatment groups and evidence of monocyte attachment (**Figure 3**). The control disk (group 1) showed parallel scratches arising from the mechanical preparation of the titanium surface as expected. This gave the titanium surface the appearance of grooves in a regular pattern and a relatively smooth appearance. Monocytes were observed on the 30-min incubation samples, but the cells were sparsely distributed on all samples at this point. The monocytes attached to the surface in group 1 were rounded and showed some early minimal signs of membrane ruffling. The sandblasting in group 2 appeared to remove the grooved appearance created by the mechanical polishing of the control samples. Craters and pits of varying dimensions were interspersed on a surface smoothed by boiling in $HCl/H_2SO_4/H_2O$ for 3 h. Cells at various stages of attachment were observed from rounded cells, to cells with more marked membrane ruffling, to cells with pseudopodial extension, to cells that exhibited marked flattening and spreading of the cell membrane. The cells appeared to be more numerous on the areas of the surface with the most marked surface features as seen with SEM. Extension of pseudopodia outwards from the cells in several of these early time samples indicated normal function. The edges of the surface scratches were softened by the surface modification in group 3 and group 4 that produced a similar surface appearance. Pits were interspersed between an even distribution of peaks and valleys following the acid etching of group 5. For group 6, the grooved pattern was greatly reduced, and a textured surface produced by treatment with $HCl/HNO_3/H_2O$ for 10 seconds followed by treatment with 70°C 5 M NaOH for 24 h, followed by in-air oxidation at 600°C for 1 h.

At 60 min incubation time and prior to activation, clumps of cells were observed attached to the group 1 surface. Extensive ruffling of the monocyte membrane and pseudopodial extension were also observed. Monocytes in group 3 exhibited a range of morphology from rounded with few pseudopodia to extensive flattening. Less ruffling of the cell membrane was noted on monocytes incubated for 60-min with group 5 than group 6 (**Figure 3**).

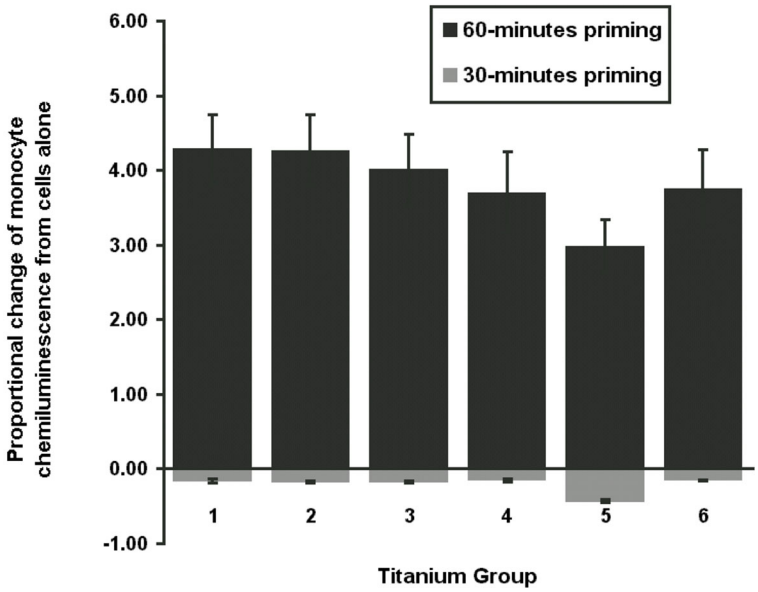

FIGURE 2 | Total chemiluminescence. Figure demonstrates the proportional change of mean monocyte chemiluminescence from cells only for 30-min or 60-min priming phase. Monocyte activation was with PMA stimulation at 10^{-5} M with luminol 10^{-6} M. CPT group 5 was significantly different to the other groups ($P < 0.001$).

Immediately following secondary stimulation with PMA, a marked flattening of cells and spreading of the cell membrane was observed for the group 1, group 4, and group 5 (**Figure 4**). A reduced level of cell membrane ruffling was observed in specimens from all other groups following PMA stimulation. At 1 h following secondary stimulation with PMA, the continued loss of surface features compared to earlier time periods was noted in all groups. This was thought to indicate the onset of apoptosis (**Figure 5**).

DISCUSSION

Surgical placement of any foreign biomaterial in the body elicits an acute inflammatory response, which is a vital biological response, which is regulated during early wound healing. that impacts later wound healing events such as osseointegration and guided bone regeneration (1). The impact of the early inflammatory phase on osseointegration of endosteal implants is potentially critical to wound healing and yet is still poorly understood. One of the factors possibly contributing to improved success rates of microrough implant surfaces is the reaction of the initial inflammatory cells populating the implant surface immediately after being placed in the surgically prepared osteotomy site. This primary cellular response in inflammation comes from neutrophils and monocytes. Monocytes constitute 5-10% of leukocytes and are responsible for releasing cytotoxic products that are essential for killing bacterial invaders, but these same molecules also destroy host tissues. Within These include singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radicals. LCL is an exquisitely sensitive method for detecting the production of reactive oxygen species. A drawback of the method is that light emission cannot be correlated with the production of a single type of oxygen metabolite, although in monocytes LCL has been shown to be dependent on extracellular myeloperoxidase release (26). As a light emission enhancer, 100 µl of luminol at a final concentration of 1 x 10^{-6} M was used based on the preliminary concentration curve experiments that determined the optimum concentration for our system. This compares to 750 µl of luminol at a final concentration of 1 x 10^{-5} M used by McNally and Bell (27) for a similar purpose in a reaction vessel containing 1 x 10^{6} human monocytes. They also found a greater LCL response, following PMA activation, with luminol than lucigenin as was found in the present study. In our preliminary studies comparing LCL output with either PMA or FMLP as secondary stimulants, we found the highest CL output with PMA, which is similar to the findings of several other studies (28, 29).

The results of this study demonstrate that incubating human peripheral blood monocytes with surface-modified CPT results in priming or inhibition of priming in a time dependent manner. Normally, primed monocytes demonstrate higher CL output when compared to unprimed cells when subsequently challenged by a standard stimulus. The 60-min incubation lead to priming of the monocytes with the highest mean proportional change of monocyte LCL in group 1, with a mean value 4.22 times cells alone, while the lowest mean change of monocyte LCL was in group 5, with value 2.98 times cells alone. Thus, all the titanium disks had a priming effect on the monocytes at 60-minutes, increasing respiratory burst activity following stimulation by PMA as compared to cells alone. The order of the mean of percentage change of

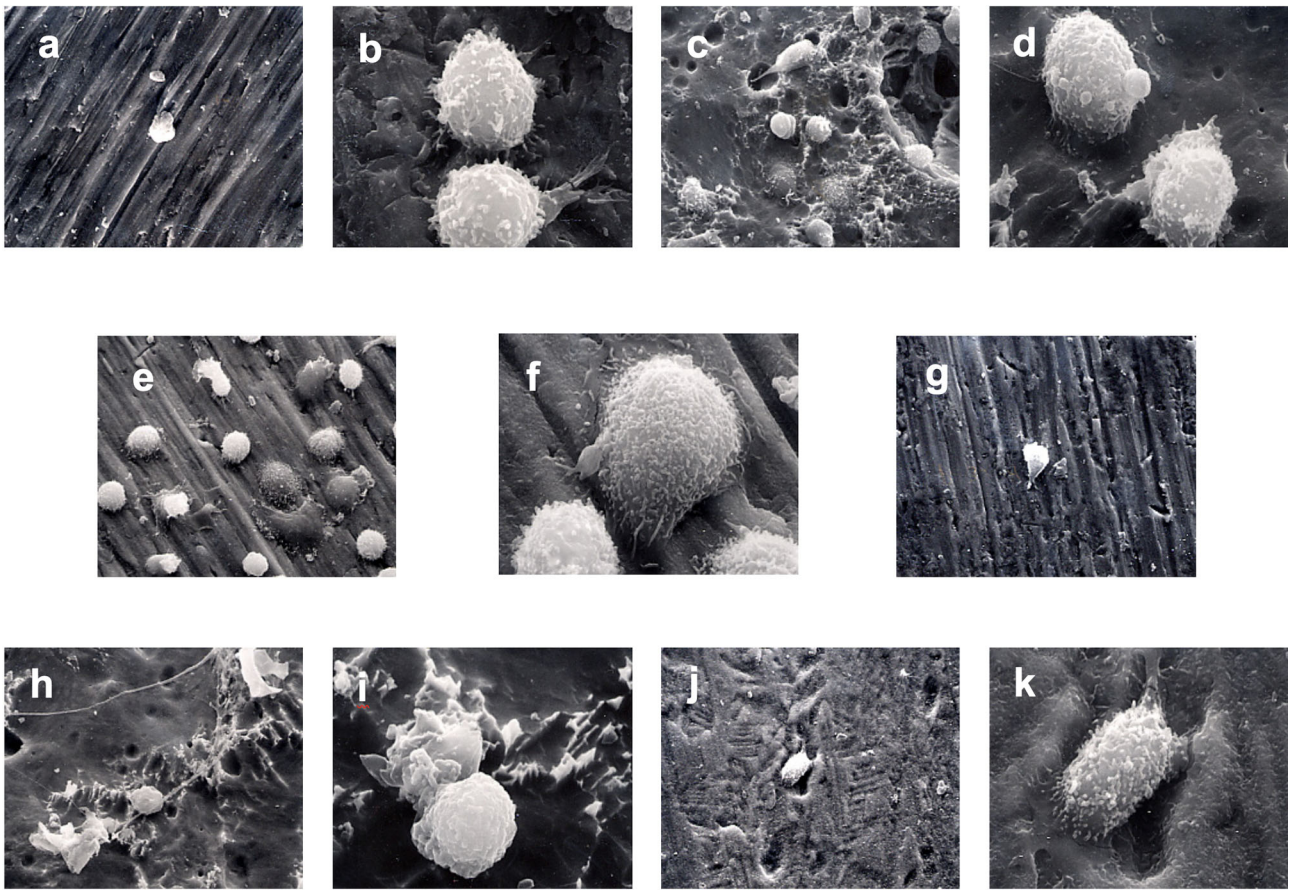

FIGURE 3 | Monocyte priming. SEM photomicrographs of monocytes incubated with surface-modified titanium prior to secondary stimulation with PMA. **(A)** 1250x Group 1 30-min incubation. **(B)** 5000x Group 1 60-min incubation. **(C)** 1250x Group 2 30-min incubation. **(D)** 5000x Group 2 60-min incubation. **(E)** 1250x Group 3 30-min incubation. **(F)** 5000x Group 3 60-min incubation. **(G)** 1250x Group 4 30-min incubation. **(H)** 1250x Group 5 30-min incubation. **(I)** 5000x Group 5 60-min incubation. **(J)** 1250x Group 6 30-min incubation. **(K)** 5000x Group 6 60-min incubation.

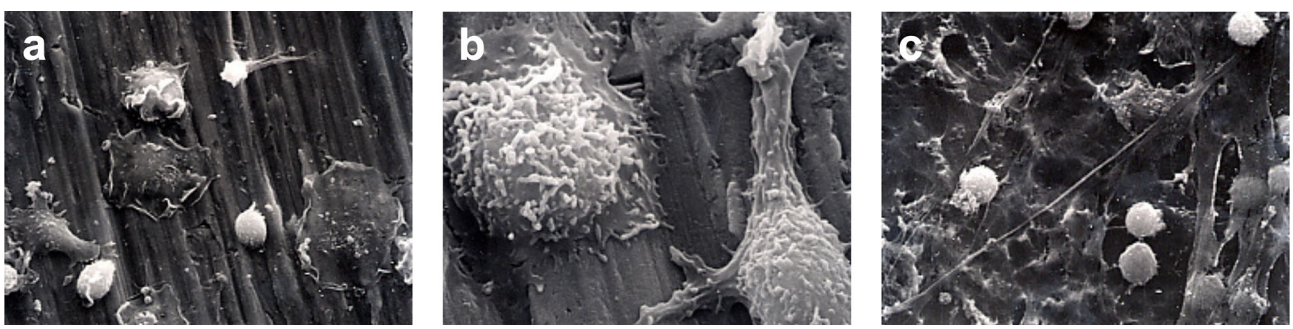

FIGURE 4 | Monocyte activation. SEM photomicrographs following 60-minutes incubation and immediately following stimulation with PMA. **(A)** 1250X magnification of Group 1. Marked flattening of cells and cell membrane spreading were observed. **(B)** 5000x magnification of Group 4. Marked ruffling of the monocyte cell membrane and pseudopodial extension was demonstrated. **(C)** 1250x magnification of Group 5.

monocyte CL among CPT groups from highest to lowest was CPT1, CPT2, CPT3, CPT6, CPT4, and CPT5. This pattern was not easily interpreted in terms of surface energy and surface roughness. However, the mean percentage change of monocyte CL in group 5 was significantly lower than the other groups, indicating that the surface characteristics of group 5 lead to significantly less priming of monocytes with 60-min incubation than the other groups. CPT group 5 had the lowest surface

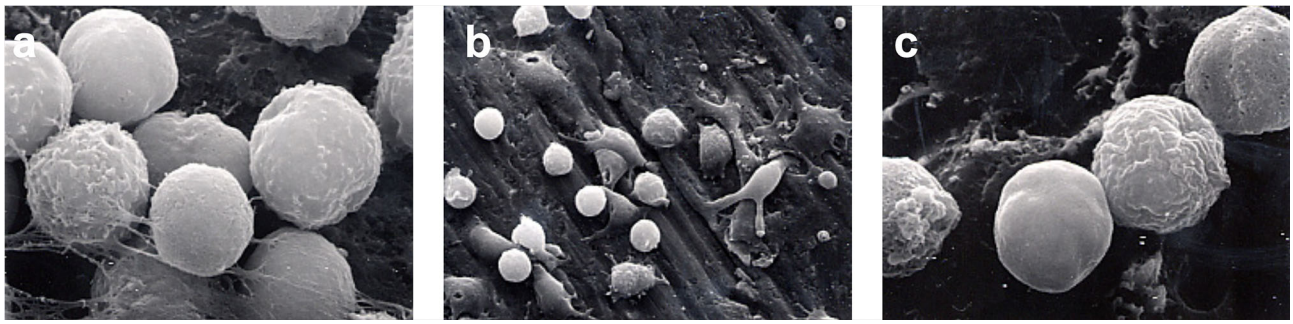

FIGURE 5 | Monocyte apoptosis. SEM photomicrographs 60-min following secondary stimulation with PMA 10^{-5} M. **(A)** 5000x magnification of Group 1. The continued loss of surface features compared to earlier time periods is noted. **(B)** 1250x magnification of Group 4. Monocytes have lost the ruffled border with many cells showing signs of apoptosis. **(C)** 5000x magnification of Group 5. Loss of surface features and onset of apoptosis is evident.

energy of all the groups, possibly suggesting that surface energy below a certain threshold level may have an effect on monocyte priming. This finding lends support to the first hypothesis. In contrast, 30-min incubation time lead to reduced LCL on secondary stimulation as compared to cells alone for all groups. In this experiment, the highest absolute mean of proportional change of monocyte LCL was in group 5, with an absolute value of 0.43, while the lowest absolute mean of proportional change of monocyte LCL was in group 4, with an absolute value of 0.15. The order of the absolute mean change of monocyte LCL among CPT groups from highest to lowest was CPT5, CPT3, CPT2, CPT1, CPT6, and CPT4. This pattern was also not easily interpreted in terms of surface energy and surface roughness. The absolute mean change of monocyte LCL in CPT group 5 was significantly higher than the other CPT groups in both analyses. This suggests that the surface characteristics of CPT group 5 lead to a reduced CL response of monocytes with 30-min incubation time. We evaluated the relationship between surface roughness and contact angle measurement to the priming effect on human monocytes because wide differences in surface morphology of CPT are known to be the result of the various surface treatments. It may be significant that the surface modification of CPT group 5 produced the surface with the greatest contact angle; that is, the lowest surface energy of all treatments and one of the highest surface roughness levels.

Wettability on the surfaces of biomaterials has been reported to affect cell attachment considerably. It is believed that microvilli and filopodia, which work advantageously at the early stage of cell attachment, are needed for cells to pass through the energy barrier between the materials and the cells themselves (29). Many reports do not give clear definitions of wettability or adequately control other relevant factors and so make comparison difficult. As for monocytes and macrophages, it has been recognized that, in general, hydrophobic particles are better taken up by macrophages than those more hydrophilic than the phagocyte's surface (30). Macrophages also attach more readily to hydrophobic surfaces, which contrasts with fibroblasts, which prefer hydrophilic surfaces (16). Within the range of surface roughness tested in the present investigation, no significant difference in LCL response of monocytes could be detected between rough or smooth titanium. With regards to surface roughness, CPT group 5 (high roughness) showed significant differences to CPT groups 1 and 4 (low roughness). However, CPT group 6 (high roughness) did not. The direct comparison of CPT groups with similar surface energies but different surface roughness, CPT group 2 versus CPT groups 1 and 4 showed no significant differences. Hence again while the overall pattern is not easy to interpret with regards to the effect of surface roughness on LCL response, these results would not support hypothesis 2 that human peripheral blood monocytes have a greater respiratory burst activation response to CPT of greater roughness than to those of lesser roughness. It may be that the ranges of surface roughness examined in the present investigation, from a minimum of 0.57 μm to a maximum of 2.38 μm, were not sufficiently diverse to show a difference. The range of roughness used in the present investigation was limited by the surface conditioning techniques used to prepare the samples, which may all be considered relatively smooth. Furthermore, the variation of the roughness within a particular group of CPT disks, shown by the standard deviation (**Table 2**), suggests the possibility of some overlap of roughness between groups. In fact, it has been proposed that the use of standard values, such as the mean roughness parameter R_a, may be inadequate to describe complex topographies such as may be produced by some of the conditioning processes used in the present study (31).

SEM examination of samples at various stages of incubation time and post secondary activation revealed a wide variation in cellular response. Cells that had spread pseudopodia and had become flattened during the priming phase were observed on the surfaces of the modified titanium disks. These characteristics, which are signs of monocyte priming, were more frequently observed at 60-min than at 30-min. However, no qualitative difference in the behavior of the monocytes to different surfaces could be detected. While many studies have demonstrated that implant surface microtopography can affect cellular response for many cell types, it is also known that implant surface composition and surface energy also affect the response (32).

Thus, directly correlating surface microroughness to a cellular response is a difficult task. Lincks et al. (1998) (33) determined the effect of chemical composition and surface roughness of CPT grade 2 and titanium alloy on MG63 osteoblast-like cells *in vitro*. They found that cell proliferation, differentiation, protein synthesis and local factor production were affected by surface roughness and composition. Enhanced differentiation of cells grown on rough surfaces (R_a 3.20-4.24 μm) compared to smooth surfaces (R_a 0.22-0.23 μm) was noted. Differences between CPT and titanium alloy of similar roughness were also noted indicating that factors other than roughness alone influence the cellular response.

Recently, Hayakawa et al. (2002) (32) placed CPT implants of either 1.3 μm or 14.1 μm surface roughness and with or without a calcium-phosphate coating in the femoral condyle of rabbits. They observed histologically the bone to implant contact up to 12 weeks and demonstrated similar bone to implant contact for rough or smooth CPT implants, but greater bone to implant contact was observed for the roughened and coated surfaces. This reinforces the rationale for continued research into surface treatments of implants that may alter the cellular response in a favorable manner. It is important to keep in mind that early events in a biologic response may explain effects seen much later in the healing process. Future studies concerned with the response of monocytes to roughened surfaces, may attempt to utilize surface treatments that produce a greater range of surface roughness while attempting to standardize other surface characteristics. This may assist in revealing any effect of surface roughness on monocyte CL response. Of course, it is likely that a minimum time is required before the effect of surface properties can be detected on cells and that this time is probably surface dependent and different for various cell types. Rich and Harris (1981) (16) found that it took between 1- and 7-days incubation for mouse peritoneal macrophages to show a preference to accumulate on rough surfaces, even though the macrophages were seen to move extensively over the surfaces during this time. This time requirement was shown to be an intrinsic property of the cells. Further, priming of the macrophages only slightly reduced the response time. This was in contrast to fibroblasts, which showed a preference for smooth surfaces within one day's incubation.

Eriksson and Nygren (2001) (34) exposed CPT sheets with a water contact angle of 11° to whole blood. They found that monocytes were present on the surface in low and rather constant amounts over the 30-min to 24-h incubation time as compared to the other cell types. The monocytes covered less than 1% of the surface in this model. Furthermore, the maximal CL response was seen at 30-min incubation, which contrasts with findings of the present investigation. However, as in the present study, a CL response different to cells alone was not noted until the secondary stimulus was added.

The SEM observation of samples at the two incubation times and following activation showed cells on the surfaces of the conditioned CPT disks that had spread pseudopodia and had become flattened during the priming phase. These characteristics, which are signs of monocyte priming, were more frequently observed at 60-min than at 30-min. However, no qualitative difference in the behavior of the monocytes to different surfaces could be detected. Leake et al. (1981) (14) observed the adhesion of alveolar and peritoneal macrophages to various surfaces within 24 h. It may be that the rounded cells seen in several of the SEM in the present study were unable to locate suitable attachments sites on the CPT surface.

SEM examination also revealed higher numbers of monocytes that appeared to be undergoing apoptosis at 60-min following secondary stimulation. This may have resulted from toxic effects of the PMA or reactive oxygen species produced during the respiratory burst. Thirdly, the surface of the CPT disks themselves may have caused this effect following prolonged incubation.

Even though these experiments were of an *in vitro* and preliminary nature, they do suggest some clinical significance. The finding that incubation of human peripheral blood monocytes with variously treated CPT surfaces can induce a time-dependent variation in the LCL response and that this response may be surface dependent, suggests that the effect of CPT surfaces on monocyte response will need to be considered in the development of dental implant surfaces. It is well documented that dental implants with rough surfaces tend to show higher bone to implant contact and greater predictability where bone quality is poor (Cochran 1999) (35). It has also been shown that rough and/or hydrophilic surfaces lead to greater macrophage induced bone resorption (15). These results seem consistent with the present investigation, which has shown that CPT surfaces can prime monocytes but that the oxidative burst of these monocytes following secondary stimulation was least on the most hydrophobic surface. This leads to the possibility that there is likely to be a range of surface roughness and surface energy values that cause an appropriate level of monocyte activation, consistent with a cellular response that results in osseointegration (36). It can be speculated that the activation of monocytes outside this range is likely to result in chronic inflammation and the formation of a fibrous capsule around the implant.

It is important to note that individual variations between blood donors could significantly impact the monocyte response. The blood samples were tested for infectious diseases but not for other chronic conditions. This could be a limitation to the current investigation, however the authors tried to control for individual variation by standardizing the use of each blood sample for each experimental run involving all of the groups. Another potential limitation of the study is the lack of characterization of the purification or polarization level of monocytes. This could have helped further clarified a source of variation of cellular reactions.

Several conclusions can be made from the present study. The data clearly show that CPT is capable of priming peripheral blood monocytes following 60-min incubation. This priming of human monocytes is time dependent, with 30-min incubation leading to an inhibition of the oxidative response compared to cells alone. Secondly, the priming effect is not dependent of surface roughness within the range of

roughness and incubation periods tested in this study. This suggests that CPT surfaces with roughness between 0.57 μm and 2.38 μm can prime human monocytes following 60-min incubation. Thirdly, CPT group 5 with a water contact angle of 72.99 degrees, lead to significantly reduced priming of human monocytes compared to the other groups tested. This suggests that the priming of human peripheral blood monocytes by CPT may be dependent on a critical threshold surface energy level.

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 studies involving human participants were reviewed and approved by Indiana University Medical School Institutional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

RD and MK contributed to the development, conceptualization and execution of the project. YO contributed to development of the materials and methods and execution of the project. KE contributed to data analysis and manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Albrektsson T, Dahl E, Embom L, Engevall S, Engquist B, Eriksson AR, et al. Osseointegrated Oral Implants. A Swedish Multicenter Study of 8139 Consecutively Inserted Nobelpharma Implants. *J Periodontol* (1988) 59:287–96. doi: 10.1902/jop.1988.59.5.287
2. Ducommun J, El Kholy K, Sutter V, Janner S, Buser D. Analysis of Trends in Implant Therapy in Surgical Specialty Clinic: An Analysis of Patients, Indications, Surgical Procedures and Early Failures. *Clin Oral Implant Res* (2019) 11:1097–106. doi: 10.1111/clr.13523
3. Brånemark P-I, Hansson BO, Adell R, Breine U, Lindstrom J, Hallen O, et al. Osseointegrated Implants in the Treatment of the Edentulous Jaw. Experience From a 10-Year Period. *Scandinavian J Plast Reconstructive Surg Supplementum* (1977) 16:1–132.
4. Kasemo B, Lausmaa J. Biomaterial and Implant Surfaces: A Surface Science Approach. *Int J Oral Maxillofac Implants* (1988) 3:247–59.
5. Kasemo B. Biocompatibility of Titanium Implants: Surface Science Aspects. *J Prosthet Dent* (1983) 49:832–7. doi: 10.1016/0022-3913(83)90359-1
6. Brunette DM, Chehroudi B. The Effects of the Surface Topography of Micromachined Titanium Substrata on Cell Behavior *In Vitro* and *In Vivo*. *J Biomechan Eng* (1999) 121:49–57. doi: 10.1115/1.2798042
7. El Kholy K, Buser D, Wittneben J-G, Bosshardt DD, Van Dyke TE, Kowolik MJ. Investigating the Response of Human Neutrophils to Hydrophilic and Hydrophobic Micro-Rough Titanium Surfaces. *Materials* (2020) 13:3421. doi: 10.3390/ma13153421
8. Buser D, Schenk RK, Steinemann S, Fiorellini JP, Fox CH, Stich H. Influence of Surface Characteristics on Bone Integration of Titanium Implants. A Histomorphometric Study in Miniature Pigs. *J Biomed Mater Res* (1991) 25:889–902. doi: 10.1002/jbm.820250708
9. Gotfredsen K, Wennerberg A, Johansson C, Skougaard LT, Hjorting-Hansen E. Anchorage of TiO_2-blasted, HA-Coated, and Machined Implants: An Experimental Study With Rabbits. *J Biomed Mater Res* (1995) 29:1223–31. doi: 10.1002/jbm.820291009
10. Sennerby L, Thomsen P, Ericson LE. Early Tissue Response to Titanium Implants Inserted in Rabbit Cortical Bone. I. Light Microscopic Observations. *J Material Science: Mater Med* (1993a) 4:240–25. doi: 10.1007/BF00122275
11. Sennerby L, Thomsen P, Ericson LE. Early Tissue Response to Titanium Implants Inserted in Rabbit Cortical Bone. II. Ultrastructural Observations. *J Material Science: Mater Med* (1993b) 4:494–502. doi: 10.1007/BF00120129
12. Park JY, Davies JE. Red Blood Cell and Platelet Interactions With Titanium Implant Surfaces. *Clin Oral Impl Res* (2000) 11:530–9. doi: 10.1034/j.1600-0501.2000.011006530.x
13. Baltadjiev G, Tsvetkova T, Novakov S, Zhelev Z. Mononuclear Phagocytic System and Bone Repair After Fractures in Man. *Folia Med (Plovdiv)* (1990) 32:17–21.
14. Leake ES, Wright MJ, Gristina AG. Comparative Study of the Adherence of Alveolar and Peritoneal Macrophages, and of Blood Monocytes to Methyl Methacrylate, Polyethylene, Stainless Steel, and Vitallium. *J Reticuloendothel Soc* (1981) 30:403–14.
15. Murray DW, Rae T, Rushton N. The Influence of the Surface Energy and Roughness of Implants on Bone Resorption. *J Bone Joint Surg Br* (1989) 71:632–7. doi: 10.1302/0301-620X.71B4.2670951
16. Rich A, Harris AK. Anomalous Preferences of Cultured Macrophages for Hydrophobic and Roughened Substrata. *J Cell Sci* (1981) 50:1–7. doi: 10.1242/jcs.50.1.1
17. Soskolne WA, Cohen S, Sennerby L, Wennerberg A, Shapira L. The Effect of Titanium Surface Roughness on the Adhesion of Monocytes and Their Secretion of TNF-a and PGE_2. *Clin Oral Impl Res* (2002) 13:86–93. doi: 10.1034/j.1600-0501.2002.130111.x
18. Labadia M, Faanes RB, Rothlein R. Role of Adherence vs. Spreading in the Induction of Membrane-Associated Interleukin-1 on Mouse Peritoneal Macrophages. *J Leukoc Biol* (1990) 48:420–5. doi: 10.1002/jlb.48.5.420
19. Lim YJ, Oshida Y, Andres CJ, Barco MT. Surface Characterizations of Variously Treated Titanium Materials. *Int J Oral Maxillofac Implants* (2001) 16:333–42.
20. Olefjord I, Hansson S. Surface Analysis of Four Dental Implant Systems. *Int J Oral Maxillofac Implants* (1993) 8:32–40.
21. Wennerberg A, Albrektsson T, Andersson B. Design and Surface Characteristics of 13 Commercially Available Oral Implant Systems. *Int J Oral Maxillofac Implants* (1993) 8:622–33.
22. Boyum A. Isolation of Mononuclear Cells and Granulocytes From Human Blood. Isolation of Mononuclear Cells by One Centrifugation, and of Granulocytes by Combining Centrifugation and Sedimentation at 1 G. *Scandinavian J Clin Lab Invest* (1968) Supplement 97:77–89.
23. Ferrante A, Thong YH. A Rapid One-Step Procedure for Purification of Mononuclear and Polymorphonuclear Leukocytes From Human Blood Using a Modification of the Hypaque-Ficoll Technique. *J Immunol Methods* (1978) 24:389–93. doi: 10.1016/0022-1759(78)90143-6
24. Ferrante A, Thong YH. Optimal Conditions for Simultaneous Purification of Mononuclear and Polymorphonuclear Leucocytes From Human Blood by the Hypaque-Ficoll Method. *J Immunol Methods* (1980) 36:109–17. doi: 10.1016/0022-1759(80)90036-8
25. Goddeeris BM, Baldwin CL, ole-MoiYoi O, Morrison WI. Improved Methods for Purification and Depletion of Monocytes From Bovine Peripheral Blood Mononuclear Cells. Functional Evaluation of Monocytes in Responses to Lectins. *J Immunol Methods* (1986) 89:165–73. doi: 10.1016/0022-1759(86)90354-6
26. Albrecht D, Jungi TW. Luminol-Enhanced Chemiluminescence Induced in Peripheral Blood-Derived Human Phagocytes: Obligatory Requirement of Myeloperoxidase Exocytosis by Monocytes. *J Leukoc Biol* (1993) 54:300–6. doi: 10.1002/jlb.54.4.300
27. McNally JA, Bell AL. Myeloperoxidase-Based Chemiluminescence of Polymorphonuclear Leukocytes and Monocytes. *J Biolumin Chemilumin* (1996) 11:99–106. doi: 10.1002/(SICI)1099-1271(199603)11:2<99::AID-BIO404>3.0.CO;2-3

28. Johansson A, Dahlgren C. Characterization of the Luminol-Amplified Light-Generating Reaction Induced in Human Monocytes. *J Leukoc Biol* (1989) 45:444–51. doi: 10.1002/jlb.45.5.444
29. Van Nerom A, Desmidt M, Ducatelle R, Haesebrouck F. Lucigenin- and Luminol-Enhanced Chemiluminescence in Turkey Monocytes. *J Biolum Chemilumin* (1997) 12:207–14. doi: 10.1002/(SICI)1099-1271(199707/08)12:4<207::AID-BIO446>3.0.CO;2-2
30. Rae T. The Macrophage Response to Implant Materials-With Special Reference to Those Used in Orthopedics. *CRC Crit Rev Biocompatibility* (1990) 2:97–126.
31. Weiland M, Sittig C, Brunette DM, Spencer ND. Measurement and Evaluation of the Chemical Composition and Topography of Titanium Implant Surfaces. In: JE Davies, editor. *Bone Engineering*, *1st*. Toronto: em squared inc (1999). p. 163–82.
32. Hayakawa T, Yoshinari M, Kiba H, Yamamoto H, Nemoto K, Jansen JA. Trabecular Bone Response to Surface Roughened and Calcium Phosphate (Ca-P) Coated Titanium Implants. *Biomaterials* (2002) 23:1025–31. doi: 10.1016/S0142-9612(01)00214-9
33. Lincks J, Boyan BD, Blanchard CR, Lohmann CH, Liu Y, Cochran DL, et al. Response of MG63 Osteoblast-Like Cells to Titanium and Titanium Alloy is Dependent on Surface Roughness and Composition. *Biomaterials* (1998) 19:2219–32. doi: 10.1016/S0142-9612(98)00144-6
34. Eriksson C, Nygren H. Adhesion Receptors of Polymorphonuclear Granulocytes on Titanium in Contact With Whole Blood. *J Lab Clin Med* (2001) 137:56–63. doi: 10.1067/mlc.2001.111470
35. Cochran DL. A Comparison of Endosseous Dental Implant Surfaces. *J Periodontol* (1999) 70:1523–39. doi: 10.1902/jop.1999.70.12.1523
36. Grinnell F. Cellular Adhesiveness and Extracellular Substrata. *Int Rev Cytol* (1978) 53:65–144. doi: 10.1016/S0074-7696(08)62241-X

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HIV-Associated Interactions Between Oral Microbiota and Mucosal Immune Cells: Knowledge Gaps and Future Directions

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Even with sustained use of antiretroviral therapy (ART), HIV-infected individuals have an increased risk of systemic comorbid conditions and oral pathologies, including opportunistic infections, oral mucosal inflammation, and gingival and periodontal diseases. The immune-mediated mechanisms that drive this increased risk, in the context of sustained viral suppression, are unclear. HIV infection, even when controlled, alters microbial communities contributing to a chronic low-grade inflammatory state that underlies these non-HIV co-morbidities. The higher prevalence of dental caries, and mucosal and periodontal inflammation reported in HIV-infected individuals on ART is often associated with differentially abundant oral microbial communities, possibly leading to a heightened susceptibility to inflammation. This mini-review highlights current gaps in knowledge regarding the microbe-mediated oral mucosal immunity with HIV infection while discussing opportunities for future research investigations and implementation of novel approaches to elucidate these gaps. Interventions targeting both inflammation and microbial diversity are needed to mitigate oral inflammation-related comorbidities, particularly in HIV-infected individuals. More broadly, additional research is needed to bolster general models of microbiome-mediated chronic immune activation and aid the development of precise microbiota-targeted interventions to reverse or mitigate adverse outcomes.

Keywords: immune, microbiome, oral, HIV, mucosal immunity

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INTRODUCTION

The impact of HIV infection on the immune system results in a high incidence of opportunistic infections, cancers, and various end-organ manifestations (1), in ways that go well beyond the direct effects of infection on target cells. Rapid loss of $CD4^+$ T cells in the gut mucosa results in loss of barrier integrity, with translocation of microbial products, including lipopolysaccharides (LPS) (2), to the systemic circulation. Such products induce high levels of inflammation, which fuels further

HIV replication, and infers damage on end organs (2–6). While this vicious circle has been documented in great detail in the gut mucosa both in humans and in pathogenic models of SIV infections in macaques (2), much less is known about the oral mucosa, despite the occurrence of oral opportunistic infections, cancers, and other oral manifestations of HIV infection. While antiretroviral therapy (ART) partially restores $CD4^+$ T-cell counts, and can suppress HIV viremia at undetectable levels, residual inflammation and disease manifestations continue to be observed in persons living with HIV (PLWH), including oral symptoms. The complex interplay between HIV and the immune system has also important effects on the microbiome, in the gut and orally, which itself may contribute to pathogenesis. Here, we review what is known about this interplay, what knowledge is lacking, and potential interventions and amelioration strategies.

Key interactions between microbiota and immune system have been reported in infectious diseases, autoimmune conditions, and cancer (7). Most of the existing research has, however, been focused on the gut, providing evidence of an altered gut microbiome in association with several diseases. In contrast, little is known about oral mucosal immunity and microbiota, particularly in PLWH. The prevalence of non-communicable diseases, including caries (8–17), mucosal inflammation (18), gingivitis (18–20), periodontal disease (21–23), and oral mucosal inflammation in general (24, 25), is higher in PLWH than in uninfected individuals, suggesting a heightened susceptibility to multifactorial chronic inflammation that would compromise the integrity of tooth-supporting tissues. The disruption of host–microbe homeostasis in oral epithelial tissues contributes to disease progression of gingival and periodontal diseases. This disruption is marked by a shift in the composition of the polymicrobial oral community to a dysbiotic and often pathogenic community, which fuels hyperactivation of the immune system and inflammatory conditions. The oral mucosa directly links oral bacteria to the bone through the teeth. The oral microbiome is complex involving several niches in the oral cavity including the saliva, tongue, supragingival and subgingival plaque, gingiva-crevicular fluid, buccal cavity, and soft (mucosal) and hard tissues. Bacteria colonizing these distinct niches are known to play a role in systemic inflammation and periodontitis, but the process by which this occurs is not known (26). The increased risk of periodontal disease in HIV-infected adults (22) and growing evidence of increased gingival inflammation in HIV-infected children (9, 19, 27–30) are likely driven by an altered or weakened immune response to oral commensals and pathogens.

IMPACT OF HIV INFECTION ON THE INTESTINAL MUCOSA

HIV infection is characterized by disruption of the intestinal immune barrier and microbial translocation of microbial products leading to immune hyperactivation (31). When HIV is transmitted *via* the gut mucosa, CD4+ T cells are lost in a short time span (2, 32). Among them, the IL-17 producing subset that is known as Th17 is selectively infected and depleted (2–6). IL-17 exerts its activity on epithelial cells, which express its receptor, eliciting several effects: 1) expression antimicrobial peptides (AMPs), including ß-defensins, S100A8/9, and lipocalin; 2) expression of cytokines and chemokines (to IL-6, G- and GM-CSF, and IL-8), which induce inflammation and activate neutrophils; and 3) tissue repair (33–35). Thus, IL-17 production, often in concomitance with IL-22, is a key contributor to tissue homeostasis and response to infection (36). If produced excessively, it can drive inflammation (37), but defects in production (or blockade with antibodies in therapy) lead to loss of mucosal integrity (34, 38). Loss of mucosal integrity results in microbial translocation, with bacterial products inducing a strong inflammatory response. Parallel observations have been made in non-human primate models infected with pathogenic SIV, while non-pathogenic SIV does not cause loss of Th17 cells and associated events (2–4, 39–41).

Besides Th17 cells, other subsets of resident lymphocytes are required for the maintenance of mucosal homeostasis (42, 43). Among them, mucosal associated invariant T (MAIT) cells are significantly decreased or dysfunctional in PLWH, and ART only enables partial recovery of these subsets (44–51).

MAIT cells are a subset of innate-like T cells known to have broad and potent antimicrobial activity in response to microbial metabolites of vitamin B2 (52–54) and innate cytokines (IL-12 and IL-18) (55). Since these stimuli are reportedly elevated following microbial translocation (56), microbial translocation is hypothesized to directly contribute to the loss of circulating MAIT cells by causing hyperactivation and exhaustion (57, 58). Increased proliferation of MAIT cells (measured by Ki67) was reported in macaques after infection with SIV or SHIV (51, 59). While no MAIT depletion was observed in infected pigtail macaques (59), decreased MAIT frequencies in peripheral blood, mesenteric lymph nodes, and BAL of SIV-infected rhesus macaques appeared to be caused by increased cell turnover and were not the result of caspase 3-mediated apoptosis (51). Factors impairing the maintenance of IL-17 secretion are also thought to contribute to the depletion of MAIT cells and other IL-17-producing subsets in HIV chronic infection (51).

Therefore, even early in HIV infection, mucosal immunity is dramatically upended. Both physical and chemical barriers (such as AMPs) are decreased. This upheaval is reflected also in the composition of the microbiome, with dysbiosis, which itself becomes a factor that might contribute to driving high levels of inflammation. Inflammation, besides damaging organs and systems, also drives HIV replication, establishing a vicious circle of inflammation/damage/HIV replication (2–6, 39–41). While early adoption of ART preserves to some degree mucosal integrity, residual inflammation is observed even in PLWH undergoing therapy (60–62).

While the rapid disappearance of gut Th17 cells in PLWH is not clearly attributed to preferential HIV infection (2, 63, 64),

it is very likely that microbial dysbiosis contributes significantly to Th17 and MAIT cell perturbation. In fact, several components of the intestinal microbiota influence cell-mediated immune response and gut dysbiosis is known to alter the homeostasis of intestinal MAIT and Th17 cells (43, 65–67). In HIV-infected macaques, gut dysbiosis resulted in an altered Th17 profile even in peripheral blood (68). Several studies suggest that PLWH have a dysbiotic gut microbiome with enrichment of Proteobacteria, *Prevotella*, Erysipelotrichaceae, and several pathobionts, and depletion of bacteria such as Lactobacillales, *Bacteriodes*, and short chain fatty acid (SCFA) producers, particularly in viremic subjects (69, 70). However, the composition of the microbiome of some ART-treated subjects was relatively similar to HIV uninfected controls in some other studies (71–76). The dysbiosis correlated with plasma levels of the inflammatory cytokine IL-6, and with activation of the kynurenine pathway, a known marker of disease progression (75). In particular, a study of a group of subjects with long-term ART controlled HIV infection showed gut microbiome dysbiosis with decreased levels of beneficial butyrate-producing taxa; the dysbiosis was associated with high levels of inflammation. Further, the gut microbiome of the PLWH was enriched in *Fusobacteria, Lactobacillus*, and Bifidobacteriales, which are typically associated with oral microbiome, possibly suggesting a loss of compartmentalization. Levels of *Prevotella*, although not differentially present in PLWH, negatively correlated with CD4+ T-cell counts (77). Dysbiosis could also be an outcome of Th17 cell depletion in the context of HIV infection. AMPs, which are produced when IL-17 binds to receptors on epithelial cells, are a key component of innate immunity on mucosa. They contribute to mucosal integrity, having co-evolved with mucosal microbiome, protecting the host against pathogenic infections (78, 79). Therefore, decreased production of IL-17 due to HIV infection is predicted to result in impaired production of AMP, loss of mucosal integrity, and dysbiosis.

ORAL MICROBIOME IN HIV

While growing evidence suggests that in the ART era, PLWH continue to experience oral inflammation-associated and/or immunodeficiency-related infections (80), few studies in comparison to the gut studies have comprehensively characterized the oral microbiota in the context of HIV exposure, infection, and treatment (26, 81–91). PLWH have increased levels of oral mucosal inflammatory markers (92, 93), as compared to HIV-uninfected subjects, suggesting likely changes in the oral bacterial composition. Similar to the gut, evidence suggests HIV infection impacts the composition of the oral microbiome with differentially abundant taxa when compared to uninfected populations; however, findings to date are varied and inconsistent. Some studies found no significant taxonomic differences (94–96), while others have reported differentially abundant taxa (81, 83–85, 87, 89, 91, 97). For the lingual microbiome, potentially pathogenic *Veillonella*, *Prevotella*, *Megasphaera*, and *Campylobacter* were enriched, while *Streptococcus* sp. were depleted (82). *Streptococcus mutans, Lactobacillus*, *Candida, Haemophilus parahaemolyticus, Actinomyces, Neisseria subflava* (91), and *Corynebacterium diphtheriae* (91) species were reported to be more abundant in saliva of PLWH individuals (82, 95, 98, 99). Several studies have observed a lower proportion of *Streptococcus mitis* in saliva of PLWH compared to the uninfected (91, 95, 98). A study on HIV-infected women found that in the infected group, the microbiome had higher representation of *Prevotella melaninogenica* and *Rothia mucilaginosa* (88). When compared to the perinatally exposed but uninfected, subgingival plaque of HIV-infected youth differed in abundance of disease-associated taxa (85). While some studies showed differences in microbiome compositions based on CD4 counts, the impact on other immune status markers was not evaluated (86, 88, 89).

Although ART has been implicated as the driver of these observed differences in the oral microbiome (87, 100), it is increasingly hard to isolate the direct impact of HIV with widespread availability of highly active ART. While data from a number of studies suggest a bidirectional relationship between pre-exposure prophylaxis (PrEP) and ART-specific regimens, and the vaginal and intestinal microbiota (101–103), little information is available with respect to the oral microbiome. A recent study (103) suggests that ART, especially non-reverse transcriptase inhibitors (NRTIs), have considerably more impact on microbiota composition and diversity in the gut, leading to dysbiosis, than in the oral cavity.

Patients with oral co-infections displayed lower abundance of *Veilonella parvula* (81, 82), while ART was associated with higher levels of *Neisseria* and *Haemophilus*. Recent 16S analyses a strong relationship between salivary microbiota and CD4 T cells in HIV-infected children, specifically a distinct oral microbial community with HIV infection and low CD4 counts (91). The abundance of *Streptococcus* and *Lactobacillus* correlated positively with CD4 counts, and negatively with viremia, suggesting an underlying protective effect of these taxa (88). However, a study of alpha microbial diversity that compared salivary and fecal microbiome in PLWH reported microbiome changes associated with ART only in the fecal microbiome (103). These inconsistencies highlight the need to standardize sample collection protocols, sample type (i.e., mucosal swab, saliva, supra- or subgingival plaque) or other experimental variables that may have biased the results (103). Aging is another important factor that was associated with increased intra-sample microbiome diversity regardless of HIV status (94). Several host factors including genetics and immune status play important roles in the colonization of pathogenic bacteria and consequently contribute to disease outcomes (86), and need to be considered when addressing this question. Further, the impact of several other confounding factors (including age, sex, dentition, oral hygiene, periodontal disease, sex, salivary flow, body mass index, diet, cigarette smoking, antibiotic use, and the type and site of specimen collection) is an important point of consideration for future studies.

While phylogenetic approaches such as 16S sequencing are able to identify taxa that are unculturable within an ecological framework, species/strain resolution is often poor and their functional roles can only be inferred. Similarly, 16S studies do not assess the composition of the mycobiome, that may interact with, and influence, the microbiome (104). Results of a study that assessed mycobiome and microbiome in smokers and non-smokers. The study reported lower alpha diversity of the mycobiome in HIV-infected smokers than HIV-infected non-smokers, while richness of the microbiome in HIV-infected smokers was less than that of uninfected smokers, suggesting complex interactions between mycobiome and microbiome in different health conditions (83). Whole genome metagenomics allow for detailed investigations into oral microbial community diversity (both intra- and inter-sample), composition and function, yet there are few available studies regarding the relationship between the function of the oral microbiota and HIV. Therefore, additional studies are needed to clarify this complexity. The synergistic impact of fungal involvement, including *Candidiasis*, on the mucosa plays an important role in mucosal immunity. This is particularly important as candidiasis was one of the most common HIV-associated oral lesions prior to HAART initiation and was often pathognomonic for disease progression. Colonization of *C. albicans* and *C. dubliniensis* are the most prominent taxa observed in high abundance in saliva of HIV infected individuals. While incidence of oral candidiasis typically declines after HAART initiation, recent evidence suggests that the impact of HIV/HAART on the mycobiome is modest but not more considerable than other factors such as sex (105).

There is a dearth of studies focused on the gene expression and metabolic function of the oral bacterial communities with HIV infection. This is critical as recent evidence suggests significant functional redundancy such that even if communities differ in abundance, there is an inherent stability in ecologic function. Bacteria of the oral microbiome release metabolites—lipids, nucleic acids, polyuronic acids, proteins, and extracellular polymeric substances and microbial production that serve several functions. Worthy of note is the production of SCFA and tryptophan. SCFA are immunomodulatory products that have several effects on the oral epithelial barrier, and could represent the link between the bacterial communities and the immune system. Butyrate is a SCFA notorious in the oral environment for its deleterious impact on the gingiva and periodontium. Some potential mechanisms by which butyrate elicits their effects include cell apoptosis and upregulation of proinflammatory cytokines and modulation of the proteins in intercellular junctions (106, 107). The activation of the tryptophan metabolism pathway by the enzyme indoleamine 2,3 dioxygenase (IDO), which is expressed in macrophages and dendritic cells, produces kynurenine and other metabolites, which have immunomodulatory effects. The kynurenine/tryptophan ratio (KTR) is considered a surrogate marker of IDO activity, and is associated with immune activation (75, 108–114). Increased KTR have been consistently reported in PLWH (75, 108–114).

IMPACT OF HIV ON ORAL MUCOSAL IMMUNITY

A predominant portion (~80%) of the oral cavity consists of oral mucosal surfaces, therefore presenting an extensive area for microbial attachment (115). Several research studies have reported mucosal immune cell dysfunction and its interaction with the oral microbiome, in the context of various chronic inflammatory diseases (116, 117). However, to our knowledge, none has comprehensively evaluated the contribution of the oral microbiota to mucosal immune perturbation in PLWH. Our understanding of the impact of HIV on the distribution and function of immune cells in the oral mucosa, the mechanism(s) for chronic oral inflammation and its role in increasing the risk for oral diseases, is limited. While there is growing evidence of a higher prevalence of oral disease in PLWH (24, 25), it is unclear how HIV, even in the context of suppressed viral replication, heightens susceptibility to oral mucosal inflammation.

Secretory immunoglobulin A (SIgA) antibodies in saliva are considered the first line of defense against pathogens present in the oral cavity. SIgA and other salivary antimicrobial systems also act against periodontopathic and cariogenic consortia by limiting adherence of pathogens and pathobionts to the mucosa (118, 119). These oral pathogens include the main cariogenic agent—*Streptococcus mutans*. SIgA plays an important role in the homeostasis of the oral microbiota as focus of much research in the last two decades has been on the development of a caries vaccine to stimulate induction of IgA responses in saliva (120, 121). In HIV+ individuals, dysregulation of CD4 T-follicular helper cell function greatly limits/impairs Ig class switching in subepithelial B cells, which results in a significant reduction of IgG and SIgA in mucosal fluids (122, 123). This decline may contribute to a perturbed composition of mucosal microbiome and to the compromise of mucosal barrier integrity.

As mentioned above, HIV infection disproportionately affects Th17 cells (2, 6, 124) (**Figure 1**). Human β defensin 2 (hBD2) is not detectable in the oral mucosa of PLWH but is robustly expressed in HIV-uninfected controls (125). This defensin is of particular interest because it binds to CCR6, a shared chemokine receptor expressed on Th17 and MAIT cells (126). We also reported that hBD2 selectively protects CCR6+ CD4 T cells from infection (127, 128). Therefore, AMP could be at the center of the mechanism underlying the effects of HIV infection on Th17 cells resulting in loss of mucosal integrity and dysbiosis. Oral epithelial barrier function and mucosal immunity clearly depends on interactions between commensal microbiota and pathogens with toll-like receptors on epithelial cells (129).

As observed in the gut, the expression of AMPs is lower in oral mucosa of HIV-infected individuals undergoing ART, as compared to HIV-seronegative controls (125). AMPs have been shown to promote targeted killing of specific pathogenic taxa (130), so with decreased levels, the immune system is further compromised. This state of impaired innate immunity could

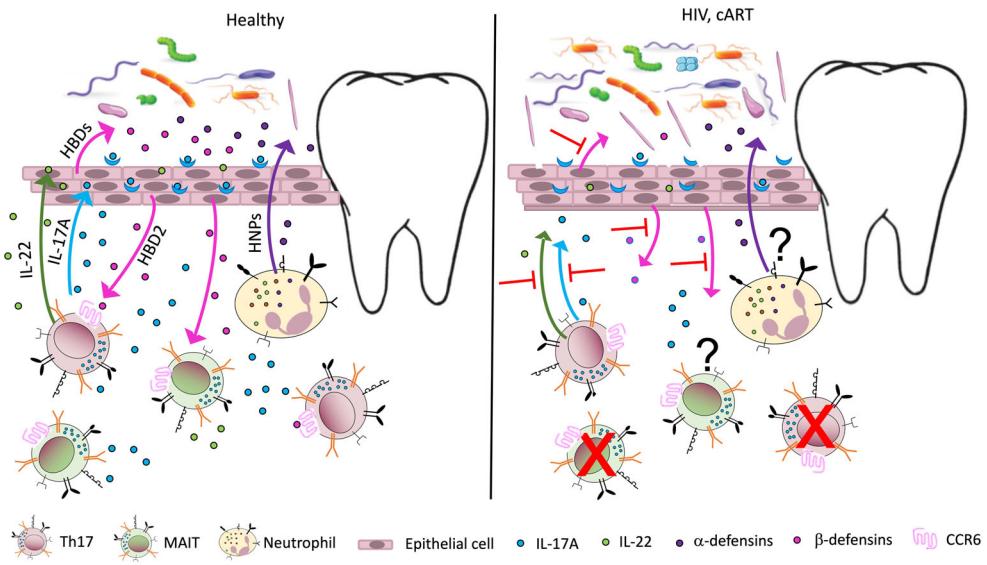

FIGURE 1 | Potential immune perturbation affecting the oral mucosa in HIV-infected individuals/ PLWH. In healthy, uninfected individuals (left panel), Th17 cells, MAIT cells, and neutrophils contribute to mucosal homeostasis by producing various soluble factors involved in mucosal integrity. Th17 and MAIT cells secrete IL-17, a cytokine with antifungal and antibacterial function, which also acts on epithelial cells to induce secretion of antimicrobial peptides called human β defensins 1–3 (HBDs). Among them, HBD2 binds the receptor CCR6, expressed by Th17 and MAIT cells, with potential cytoprotective effects. Th17 cells also produce IL-22, important for tissue repair, while neutrophils are responsible for the secretion of another class of antimicrobial peptides, the human α defensins or human neutrophil peptides (HNPs). The antimicrobial peptides contribute to the homeostasis of the mucosal microbiome, promoting oral colonization of advantageous bacterial species. In PLWH (right panel), a large number of Th17 cells in the gut mucosa are lost due to active infection. The number of MAIT cells also declines in peripheral blood, possibly due to activation induced cell death. A decline in Th17 and MAIT cells may occur to some extent in the oral mucosa, leading to decreased levels of IL-17, IL-22, and HBDs. This imbalance may contribute to increased inflammation and perturbed microbiome in the oral mucosa (dysbiosis), increasing the risk of oral disease. The effects of antiretroviral therapy (cART) on oral inflammation and dysbiosis are unclear.

increase the risk of oral mucosal pathologies such as gingival and periodontal inflammation. Microbial changes observed after ART administration include decrease in salivary *Aggregatibacter*, *Prevotella*, and *Haemophilus* which could in turn drive pathogenesis or facilitate colonization of taxa that have been implicated in periodontal disease (*Porphyromonas, Prevotella melaninogenica, Rothia mucilaginosa* and *Fusobacterium* in saliva, and *Rothia dentocariosa, Fusobacterium, Streptococcus*, and *Prevotella* in plaque) (87, 103). As a keystone pathogen, *P. gingivalis* impairs host immune responses and represents a necessary but not sufficient microbe for development of periodontitis. These taxa should be considered in the pursuit of developing treatments to minimize HIV-associated periodontal disease.

Studies focused on identifying early immunology and microbiota differences that could lead PLWH to an increased susceptibility to chronic inflammatory conditions are needed. This data is highly relevant to human health, addressing the role of the oral microbiota on immune cell response. By targeting AMPs and specific immune cells, which are known drivers of immunomodulation with established relevance and therapeutic potential, there would be an improved understanding of how the oral microbiota influences immune pathology, informing novel interventions for a wide range of oral diseases including mucosal infections and cancer.

FUTURE INVESTIGATIONS AND THERAPEUTIC APPROACHES

Our understanding of the interaction between oral microbiota and oral mucosal immune cells is still evolving. Significant gaps remain with respect to mechanisms of influence by microbiota on immune homoeostasis, and *vice versa*. Given the bidirectional relationship between oral microbiota and mucosal immunity (97), understanding the functions of microbes involved in influencing immune compartments, their physio-pathologic consequences, and contribution to oral disease pathology is essential to inform preventive and therapeutic approaches. This complex relationship demonstrates a clear need for continued investigation in both animal and human studies. Investigating the interplay between immune cell subsets and bacterial communities would inform strategies to improve and facilitate mucosal homeostasis.

Studies aimed at identifying early immunological and microbial features with the potential to increase the risk of chronic inflammatory conditions in PLWH are needed. In particular, understanding the interplay between oral microbiota and mucosal immunity may identify targets with therapeutic potential, thus informing novel interventions for a wide range of oral diseases, including mucosal infections and cancer. While there is evidence of SIgA in the oral mucosa with

HIV, its unique molecular properties and interactions with pathogens and microbial metabolites should be incorporated in further investigations of the oral mucosal immunity (131). The specific impact of HIV on oral mucosal immunity has significant implications for specific sub-groups and risk populations such at sero-discordant couples, men who have sex with men (MSM), the aging, and growing children and adolescents.

Given the gaps in knowledge, characterizing features and functions of the oral microbiota associated with HIV infection would bring us closer to understanding the interplay between the oral microbiota and oral mucosal immunity. Multi-dimensional and multi-parametric approaches are needed to investigate microbe-mediated interactions as well as identify the microbial properties and immune parameters key for oral mucosal homeostasis. Such approaches will provide insight into how the oral microbiota could be used to mitigate immune perturbation in HIV.

Probiotic approaches to prevent oral diseases have been in previously spotlighted. An example in caries disease treatment is the displacement of native *S. mutans* strains with *S. mutans* strains engineered to have low pathogenicity (132, 133). While the results support the efficacy of these strains as anti-caries probiotics, further studies in humans are required. Future high-quality randomized controlled clinical trials that demonstrate the efficacy of probiotics (134, 135), antimicrobial agents and procedures on oral immune functions, will expand the current paradigm focused on intestinal bacteria by comprehensively studying microbe-mediated immune cell responses relative to oral bacteria. Understanding how the interaction between immune system and microbiota contributes to co-morbidities would provide additional targets for intervention and drive the success of future clinical trials. Research focused on bolstering general models of microbiome-mediated chronic immune activation and aiding the development of precise microbiota-targeted interventions to reverse chronic inflammation are needed. Cytological experiments and metagenome and transcriptome analyses will further characterize the biological processes and the molecular changes of specific oral bacteria. Results from future research studies are likely to inform preventive and therapeutic interventions. Interventions targeting both inflammation and microbial diversity are needed to reduce the risk of oral inflammation-related comorbidities, particularly in PLWH (84), high-risk populations such as MSM, and even more critical in developmental phases in children where appropriate immune training and maturation has far-reaching complications (136).

CONCLUSION AND PERSPECTIVES

In this review, we have discussed the current status of research on gut microbiome and HIV and reviewed recent advances in our understanding of the interaction between the oral microbiota and mucosal immune system in PLWH. Many studies of the oral microbiota suggest that individual or singular pathogens are not observed as differentially abundant in classic oral diseases such as caries or periodontitis. Perturbations among relatively less-abundant microbes appear to drive dysbiosis. Review of studies highlight an altered pathological status of the microbial communities and the immune systems even with ART. However, much work is required for a clearer understanding of the mechanisms of interaction between oral bacteria and specific T-cell subsets and their function. Therefore, in the future, it is important to focus our attention on the how to approach therapeutically dysbiosis, and/or its metabolic/inflammatory consequences, to ameliorate oral symptoms and standard of living of PLWH.

AUTHOR CONTRIBUTIONS

MC conceived the mini-review. CC developed the figure and AG-D provided guidance in direction. All authors edited and approved submission of the final version paper.

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REFERENCES

1. Gruffaz M, Zhang T, Marshall V, Goncalves P, Ramaswami R, Labo N, et al. Signatures of Oral Microbiome in HIV-Infected Individuals With Oral Kaposi's Sarcoma and Cell-Associated KSHV DNA. *PLoS Pathog* (2020) 16 (1):e1008114. doi: 10.1371/journal.ppat.1008114
2. Brenchley JM, Paiardini M, Knox KS, Asher AI, Cervasi B, Asher TE, et al. Differential Th17 CD4 T-Cell Depletion in Pathogenic and Nonpathogenic Lentiviral Infections. *Blood* (2008) 112(7):2826–35. doi: 10.1182/blood-2008-05-159301
3. Cecchinato V, Trindade CJ, Laurence A, Heraud JM, Brenchley JM, Ferrari MG, et al. Altered Balance Between Th17 and Th1 Cells at Mucosal Sites Predicts AIDS Progression in Simian Immunodeficiency Virus-Infected Macaques. *Mucosal Immunol* (2008) 1(4):279–88. doi: 10.1038/mi.2008.14
4. Schuetz A, Deleage C, Sereti I, Rerknimitr R, Phanuphak N, Phuang-Ngern Y, et al. Initiation of ART During Early Acute HIV Infection Preserves Mucosal Th17 Function and Reverses HIV-Related Immune Activation. *PLoS Pathog* (2014) 10(12):e1004543. doi: 10.1371/journal.ppat.1004543
5. Ryan ES, Micci L, Fromentin R, Paganini S, McGary CS, Easley K, et al. Loss of Function of Intestinal IL-17 and IL-22 Producing Cells Contributes to Inflammation and Viral Persistence in SIV-Infected Rhesus Macaques. *PLoS Pathog* (2016) 12(2):e1005412. doi: 10.1371/journal.ppat.1005412
6. El Hed A, Khaitan A, Kozhaya L, Manel N, Daskalakis D, Borkowsky W, et al. Susceptibility of Human Th17 Cells to Human Immunodeficiency Virus and Their Perturbation During Infection. *J Infect Dis* (2010) 201 (6):843–54. doi: 10.1086/651021
7. Lazar V, Ditu LM, Pircalabioru GG, Gheorghe I, Curutiu C, Holban AM, et al. Aspects of Gut Microbiota and Immune System Interactions in Infectious Diseases, Immunopathology, and Cancer. *Front Immunol* (2018) 9:1830. doi: 10.3389/fimmu.2018.01830
8. Rajonson N, Meless D, Ba B, Faye M, Diby JS, N'Zore S, et al. High Prevalence of Dental Caries Among HIV-Infected Children in West Africa

Compared to Uninfected Siblings. *J Public Health Dent* (2017) 77(3):234–43. doi: 10.1111/jphd.12203
9. Coker M, El-Kamary SS, Enwonwu C, Blattner W, Langenberg P, Mongodin E, et al. Perinatal HIV Infection and Exposure and Their Association With Dental Caries in Nigerian Children. *Pediatr Infect Dis J* (2018) 37(1):59–65. doi: 10.1097/INF.0000000000001702
10. Beena JP. Prevalence of Dental Caries and Its Correlation With the Immunologic Profile in HIV-Infected Children on Antiretroviral Therapy. *Eur J Paediatr Dent* (2011) 12(2):87–90.
11. Joosab Z, Yengopal V, Nqcobo CB. Caries Prevalence Among HIV-Infected Children Between Four and Ten Years Old at a Paediatric Virology Out-Patients Ward in Johannesburg, Gauteng Province, South Africa. *SADJ* (2012) 67(7):314–7.
12. Obileye MF, Agbelusi GA, Orenuga OO, Temiye EO. Dental Caries Status of HIV Infected Children in Nigeria. *Nig Q J Hosp Med* (2009) 19(4):210–3. doi: 10.4314/nqjhm.v19i4.54530
13. Tofsky N, Nelson EM, Lopez RN, Catalanotto FA, Fine DH, Katz RV. Dental Caries in HIV-Infected Children Versus Household Peers: Two-Year Findings. *Pediatr Dent* (2000) 22(3):207–14.
14. Hicks MJ, Flaitz CM, Carter AB, Cron SG, Rossmann SN, Simon CL, et al. Dental Caries in HIV-Infected Children: A Longitudinal Study. *Pediatr Dent* (2000) 22(5):359–64.
15. Madigan A, Murray PA, Houpt M, Catalanotto F, Feuerman M. Caries Experience and Cariogenic Markers in HIV-Positive Children and Their Siblings. *Pediatr Dent* (1996) 18(2):129–36.
16. Valdez IH, Pizzo PA, Atkinson JC. Oral Health of Pediatric AIDS Patients: A Hospital-Based Study. *ASDC J Dent Child* (1994) 61(2):114–8.
17. Howell RB, Jandinski J, Palumbo P, Shey Z, Houpt M. Dental Caries in HIV-Infected Children. *Pediatr Dent* (1992) 14(6):370–1.
18. Ottria L, Lauritano D, Oberti L, Candotto V, Cura F, Tagliabue A, et al. Prevalence of HIV-Related Oral Manifestations and Their Association With HAART and CD4+ T Cell Count: A Review. *J Biol Regul Homeost Agents* (2018) 32(2 Suppl 1):51–9.
19. Vieira AR, de Souza IP, Modesto A, Castro GF, Vianna R. Gingival Status of HIV+ Children and the Correlation With Caries Incidence and Immunologic Profile. *Pediatr Dent* (1998) 20(3):169–72.
20. Lauritano D, Moreo G, Oberti L, Lucchese A, Di Stasio D, Conese M, et al. Oral Manifestations in HIV-Positive Children: A Systematic Review. *Pathogens* (2020) 9(2):88. doi: 10.3390/pathogens9020088
21. Ryder MI, Shiboski C, Yao TJ, Moscicki AB. Current Trends and New Developments in HIV Research and Periodontal Diseases. *Periodontol 2000* (2020) 82(1):65–77. doi: 10.1111/prd.12321
22. Mataftsi M, Skoura L, Sakellari D. HIV Infection and Periodontal Diseases: An Overview of the Post-HAART Era. *Oral Dis* (2011) 17(1):13–25. doi: 10.1111/j.1601-0825.2010.01727.x
23. Moscicki AB, Yao TJ, Russell JS, Farhat S, Scott M, Magpantay L, et al. Biomarkers of Oral Inflammation in Perinatally HIV-Infected and Perinatally HIV-Exposed, Uninfected Youth. *J Clin Periodontol* (2019) 46 (11):1072–82. doi: 10.1111/jcpe.13179
24. Malamud D, Wahl SM. The Mouth: A Gateway or a Trap for HIV? *AIDS* (2010) 24(1):5–16. doi: 10.1097/QAD.0b013e328333525f
25. Bajpai S, Pazare AR. Oral Manifestations of HIV. *Contemp Clin Dent* (2010) 1(1):1–5. doi: 10.4103/0976-237X.62510
26. Fulcher JA. Is the Oral Microbiome Important in HIV-Associated Inflammation? *mSphere* (2020) 5(1):e00034–20. doi: 10.1128/mSphere.00034-20
27. Schoen DH, Murray PA, Nelson E, Catalanotto FA, Katz RV. Fine DH. A Comparison of Periodontal Disease in HIV-Infected Children and Household Peers: A Two Year Report. *Pediatr Dent* (2000) 22(5):365–9.
28. dos Santos Pinheiro R, Franca TT, Ribeiro CM, Leao JC, de Souza IP, Castro GF. Oral Manifestations in Human Immunodeficiency Virus Infected Children in Highly Active Antiretroviral Therapy Era. *J Oral Pathol Med* (2009) 38(8):613–22. doi: 10.1111/j.1600-0714.2009.00789.x
29. Shiboski CH, Yao TJ, Russell JS, Ryder MI, Van Dyke RB, Seage GR 3rd, et al. The Association Between Oral Disease and Type of Antiretroviral Therapy Among Perinatally HIV-Infected Youth. *AIDS* (2018) 32(17):2497–505. doi: 10.1097/QAD.0000000000001965
30. Blignaut E, Rossouw TM, Becker PJ, Mavuso DS, Feucht UD. Gingival Recession and Localized Aggressive Periodontitis Among HIV-Infected Children and Adolescents Receiving Antiretroviral Therapy. *Pediatr Infect Dis J* (2019) 38(6):e112–5. doi: 10.1097/INF.0000000000002166
31. Sandler NG, Douek DC. Microbial Translocation in HIV Infection: Causes, Consequences and Treatment Opportunities. *Nat Rev Microbiol* (2012) 10 (9):655–66. doi: 10.1038/nrmicro2848
32. Douek DC, Brenchley JM, Betts MR, Ambrozak DR, Hill BJ, Okamoto Y, et al. HIV Preferentially Infects HIV-Specific CD4+ T Cells. *Nature* (2002) 417(6884):95–8. doi: 10.1038/417095a
33. Li X, Bechara R, Zhao J, McGeachy MJ, Gaffen SL. IL-17 Receptor-Based Signaling and Implications for Disease. *Nat Immunol* (2019) 20(12):1594–602. doi: 10.1038/s41590-019-0514-y
34. Stockinger B, Omenetti S. The Dichotomous Nature of T Helper 17 Cells. *Nat Rev Immunol* (2017) 17(9):535–44. doi: 10.1038/nri.2017.50
35. Conti HR, Shen F, Nayyar N, Stocum E, Sun JN, Lindemann MJ, et al. Th17 Cells and IL-17 Receptor Signaling Are Essential for Mucosal Host Defense Against Oral Candidiasis. *J Exp Med* (2009) 206(2):299–311. doi: 10.1084/jem.20081463
36. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 Are Coexpressed by Th17 Cells and Cooperatively Enhance Expression of Antimicrobial Peptides. *J Exp Med* (2006) 203(10):2271–9. doi: 10.1084/jem.20061308
37. Teunissen MB, Koomen CW, de Waal Malefyt R, Wierenga EA, Bos JD. Interleukin-17 and Interferon-Gamma Synergize in the Enhancement of Proinflammatory Cytokine Production by Human Keratinocytes. *J Invest Dermatol* (1998) 111(4):645–9. doi: 10.1046/j.1523-1747.1998.00347.x
38. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PD, et al. Secukinumab, a Human Anti-IL-17A Monoclonal Antibody, for Moderate to Severe Crohn's Disease: Unexpected Results of a Randomised, Double-Blind Placebo-Controlled Trial. *Gut* (2012) 61 (12):1693–700. doi: 10.1136/gutjnl-2011-301668
39. Raffatellu M, Santos RL, Verhoeven DE, George MD, Wilson RP, Winter SE, et al. Simian Immunodeficiency Virus-Induced Mucosal Interleukin-17 Deficiency Promotes Salmonella Dissemination From the Gut. *Nat Med* (2008) 14(4):421–8. doi: 10.1038/nm1743
40. Klatt NR, Estes JD, Sun X, Ortiz AM, Barber JS, Harris LD, et al. Loss of Mucosal CD103+ DCs and IL-17+ and IL-22+ Lymphocytes Is Associated With Mucosal Damage in SIV Infection. *Mucosal Immunol* (2012) 5(6):646–57. doi: 10.1038/mi.2012.38
41. Hartigan-O'Connor DJ, Abel K, Van Rompay KK, Kanwar B, McCune JM. SIV Replication in the Infected Rhesus Macaque Is Limited by the Size of the Preexisting TH17 Cell Compartment. *Sci Transl Med* (2012) 4(136):136ra69. doi: 10.1126/scitranslmed.3003941
42. Wacleche VS, Landay A, Routy JP, Ancuta P. The Th17 Lineage: From Barrier Surfaces Homeostasis to Autoimmunity, Cancer, and HIV-1 Pathogenesis. *Viruses* (2017) 9(10):303. doi: 10.3390/v9100303
43. Amini A, Pang D, Hackstein CP, Klenerman P. MAIT Cells in Barrier Tissues: Lessons From Immediate Neighbors. *Front Immunol* (2020) 11:584521. doi: 10.3389/fimmu.2020.584521
44. Macal M, Sankaran S, Chun TW, Reay E, Flamm J, Prindiville TJ, et al. Effective CD4+ T-Cell Restoration in Gut-Associated Lymphoid Tissue of HIV-Infected Patients Is Associated With Enhanced Th17 Cells and Polyfunctional HIV-Specific T-Cell Responses. *Mucosal Immunol* (2008) 1 (6):475–88. doi: 10.1038/mi.2008.35
45. Mehandru S, Poles MA, Tenner-Racz K, Jean-Pierre P, Manuelli V, Lopez P, et al. Lack of Mucosal Immune Reconstitution During Prolonged Treatment of Acute and Early HIV-1 Infection. *PLoS Med* (2006) 3(12):e484. doi: 10.1371/journal.pmed.0030484
46. Caruso MP, Falivene J, Holgado MP, Zurita DH, Laufer N, Castro C, et al. Impact of HIV-ART on the Restoration of Th17 and Treg Cells in Blood and Female Genital Mucosa. *Sci Rep* (2019) 9(1):1978. doi: 10.1038/s41598-019-38547-1
47. Leeansyah E, Ganesh A, Quigley MF, Sonnerborg A, Andersson J, Hunt PW, et al. Activation, Exhaustion, and Persistent Decline of the Antimicrobial MR1-Restricted MAIT-Cell Population in Chronic HIV-1 Infection. *Blood* (2013) 121(7):1124–35. doi: 10.1182/blood-2012-07-445429
48. Eberhard JM, Hartjen P, Kummer S, Schmidt RE, Bockhorn M, Lehmann C, et al. CD161+ MAIT Cells Are Severely Reduced in Peripheral Blood and Lymph Nodes of HIV-Infected Individuals Independently of Disease

Progression. *PLoS One* (2014) 9(11):e111323. doi: 10.1371/journal.pone.0111323
49. Khaitan A, Kilberg M, Kravietz A, Ilmet T, Tastan C, Mwamzuka M, et al. HIV-Infected Children Have Lower Frequencies of CD8+ Mucosal-Associated Invariant T (MAIT) Cells That Correlate With Innate, Th17 and Th22 Cell Subsets. *PLoS One* (2016) 11(8):e0161786. doi: 10.1371/journal.pone.0161786
50. Spaan M, Hullegie SJ, Beudeker BJ, Kreefft K, van Oord GW, Groothuismink ZM, et al. Frequencies of Circulating MAIT Cells Are Diminished in Chronic HCV, HIV and HCV/HIV Co-Infection and Do Not Recover During Therapy. *PLoS One* (2016) 11(7):e0159243. doi: 10.1371/journal.pone.0159243
51. Vinton C, Wu F, Rossjohn J, Matsuda K, McCluskey J, Hirsch V, et al. Mucosa-Associated Invariant T Cells Are Systemically Depleted in Simian Immunodeficiency Virus-Infected Rhesus Macaques. *J Virol* (2016) 90 (9):4520–9. doi: 10.1128/JVI.02876-15
52. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, et al. MR1 Presents Microbial Vitamin B Metabolites to MAIT Cells. *Nature* (2012) 491 (7426):717–23. doi: 10.1038/nature11605
53. Eckle SB, Corbett AJ, Keller AN, Chen Z, Godfrey DI, Liu L, et al. Recognition of Vitamin B Precursors and Byproducts by Mucosal Associated Invariant T Cells. *J Biol Chem* (2015) 290(51):30204–11. doi: 10.1074/jbc.R115.685990
54. Corbett AJ, Eckle SB, Birkinshaw RW, Liu L, Patel O, Mahony J, et al. T-Cell Activation by Transitory Neo-Antigens Derived From Distinct Microbial Pathways. *Nature* (2014) 509(7500):361–5. doi: 10.1038/nature13160
55. Xiao X, Cai J. Mucosal-Associated Invariant T Cells: New Insights Into Antigen Recognition and Activation. *Front Immunol* (2017) 8:1540. doi: 10.3389/fimmu.2017.01540
56. Klatt NR, Funderburg NT, Brenchley JM. Microbial Translocation, Immune Activation, and HIV Disease. *Trends Microbiol* (2013) 21(1):6–13. doi: 10.1016/j.tim.2012.09.001
57. Cosgrove C, Ussher JE, Rauch A, Gartner K, Kurioka A, Huhn MH, et al. Early and Nonreversible Decrease of CD161++ /MAIT Cells in HIV Infection. *Blood* (2013) 121(6):951–61. doi: 10.1182/blood-2012-06-436436
58. Fernandez CS, Amarasena T, Kelleher AD, Rossjohn J, McCluskey J, Godfrey DI, et al. MAIT Cells Are Depleted Early But Retain Functional Cytokine Expression in HIV Infection. *Immunol Cell Biol* (2015) 93(2):177–88. doi: 10.1038/icb.2014.91
59. Juno JA, Wragg KM, Amarasena T, Meehan BS, Mak JYW, Liu L, et al. MAIT Cells Upregulate Alpha4beta7 in Response to Acute Simian Immunodeficiency Virus/Simian HIV Infection But Are Resistant to Peripheral Depletion in Pigtail Macaques. *J Immunol* (2019) 202(7):2105–20. doi: 10.4049/jimmunol.1801405
60. Hunt PW, Martin JN, Sinclair E, Bredt B, Hagos E, Lampiris H, et al. T Cell Activation Is Associated With Lower CD4+ T Cell Gains in Human Immunodeficiency Virus-Infected Patients With Sustained Viral Suppression During Antiretroviral Therapy. *J Infect Dis* (2003) 187 (10):1534–43. doi: 10.1086/374786
61. Kalayjian RC, Machekano RN, Rizk N, Robbins GK, Gandhi RT, Rodriguez BA, et al. Pretreatment Levels of Soluble Cellular Receptors and Interleukin-6 Are Associated With HIV Disease Progression in Subjects Treated With Highly Active Antiretroviral Therapy. *J Infect Dis* (2010) 201(12):1796–805. doi: 10.1086/652750
62. Sauce D, Larsen M, Fastenackels S, Pauchard M, Ait-Mohand H, Schneider L, et al. HIV Disease Progression Despite Suppression of Viral Replication Is Associated With Exhaustion of Lymphopoiesis. *Blood* (2011) 117(19):5142–51. doi: 10.1182/blood-2011-01-331306
63. Prendergast A, Prado JG, Kang YH, Chen F, Riddell LA, Luzzi G, et al. HIV-1 Infection Is Characterized by Profound Depletion of CD161+ Th17 Cells and Gradual Decline in Regulatory T Cells. *AIDS* (2010) 24(4):491–502. doi: 10.1097/QAD.0b013e3283344895
64. Gosselin A, Monteiro P, Chomont N, Diaz-Griffero F, Said EA, Fonseca S, et al. Peripheral Blood CCR4+CCR6+ and CXCR3+CCR6+CD4+ T Cells Are Highly Permissive to HIV-1 Infection. *J Immunol* (2010) 184(3):1604–16. doi: 10.4049/jimmunol.0903058
65. Gaboriau-Routhiau V, Rakotobe S, Lecuyer E, Mulder I, Lan A, Bridonneau C, et al. The Key Role of Segmented Filamentous Bacteria in the Coordinated Maturation of Gut Helper T Cell Responses. *Immunity* (2009) 31(4):677–89. doi: 10.1016/j.immuni.2009.08.020
66. Krause JL, Schape SS, Schattenberg F, Muller S, Ackermann G, Rolle-Kampczyk UE, et al. The Activation of Mucosal-Associated Invariant T (MAIT) Cells Is Affected by Microbial Diversity and Riboflavin Utilization In Vitro. *Front Microbiol* (2020) 11:755. doi: 10.3389/fmicb.2020.00755
67. Merlini E, Cerrone M, van Wilgenburg B, Swadling L, Cannizzo ES, d'Arminio Monforte A, et al. Association Between Impaired Valpha7.2 +CD161++CD8+ (MAIT) and Valpha7.2+CD161-CD8+ T-Cell Populations and Gut Dysbiosis in Chronically HIV- and/or HCV-Infected Patients. *Front Microbiol* (2019) 10:1972. doi: 10.3389/fmicb.2019.01972
68. Hensley-McBain T, Zevin AS, Manuzak J, Smith E, Gile J, Miller C, et al. Effects of Fecal Microbial Transplantation on Microbiome and Immunity in Simian Immunodeficiency Virus-Infected Macaques. *J Virol* (2016) 90 (10):4981–9. doi: 10.1128/JVI.00099-16
69. Dillon SM, Frank DN, Wilson CC. The Gut Microbiome and HIV-1 Pathogenesis: A Two-Way Street. *AIDS* (2016) 30(18):2737–51. doi: 10.1097/QAD.0000000000001289
70. Dubourg G, Surenaud M, Levy Y, Hue S, Raoult D. Microbiome of HIV-Infected People. *Microb Pathog* (2017) 106:85–93. doi: 10.1016/j.micpath.2016.05.015
71. Nowak P, Troseid M, Avershina E, Barqasho B, Neogi U, Holm K, et al. Gut Microbiota Diversity Predicts Immune Status in HIV-1 Infection. *AIDS* (2015) 29(18):2409–18. doi: 10.1097/QAD.0000000000000869
72. Dillon SM, Lee EJ, Kotter CV, Austin GL, Dong Z, Hecht DK, et al. An Altered Intestinal Mucosal Microbiome in HIV-1 Infection Is Associated With Mucosal and Systemic Immune Activation and Endotoxemia. *Mucosal Immunol* (2014) 7(4):983–94. doi: 10.1038/mi.2013.116
73. Mutlu EA, Keshavarzian A, Losurdo J, Swanson G, Siewe B, Forsyth C, et al. A Compositional Look at the Human Gastrointestinal Microbiome and Immune Activation Parameters in HIV Infected Subjects. *PLoS Pathog* (2014) 10(2):e1003829. doi: 10.1371/journal.ppat.1003829
74. Lozupone CA, Li M, Campbell TB, Flores SC, Linderman D, Gebert MJ, et al. Alterations in the Gut Microbiota Associated With HIV-1 Infection. *Cell Host Microbe* (2013) 14(3):329–39. doi: 10.1016/j.chom.2013.08.006
75. Vujkovic-Cvijin I, Dunham RM, Iwai S, Maher MC, Albright RG, Broadhurst MJ, et al. Dysbiosis of the Gut Microbiota Is Associated With HIV Disease Progression and Tryptophan Catabolism. *Sci Transl Med* (2013) 5(193):193ra91. doi: 10.1126/scitranslmed.3006438
76. McHardy IH, Li X, Tong M, Ruegger P, Jacobs J, Borneman J, et al. HIV Infection Is Associated With Compositional and Functional Shifts in the Rectal Mucosal Microbiota. *Microbiome* (2013) 1(1):26. doi: 10.1186/2049-2618-1-26
77. Rhoades N, Mendoza N, Jankeel A, Sureshchandra S, Alvarez AD, Doratt B, et al. Altered Immunity and Microbial Dysbiosis in Aged Individuals With Long-Term Controlled HIV Infection. *Front Immunol* (2019) 10:463. doi: 10.3389/fimmu.2019.00463
78. Semple CA, Taylor K, Eastwood H, Barran PE, Dorin JR. Beta-Defensin Evolution: Selection Complexity and Clues for Residues of Functional Importance. *Biochem Soc Trans* (2006) 34(Pt 2):257–62. doi: 10.1042/BST0340257
79. Gorr SU, Abdolhosseini M. Antimicrobial Peptides and Periodontal Disease. *J Clin Periodontol* (2011) 38(Suppl 11):126–41. doi: 10.1111/j.1600-051X.2010.01664.x
80. Greenspan NS. Design Challenges for HIV-1 Vaccines Based on Humoral Immunity. *Front Immunol* (2014) 5:335. doi: 10.3389/fimmu.2014.00335
81. Yang L, Dunlap DG, Qin S, Fitch A, Li K, Koch CD, et al. Alterations in Oral Microbiota in HIV Are Related to Decreased Pulmonary Function. *Am J Respir Crit Care Med* (2020) 201(4):445–57. doi: 10.1164/rccm.201905-1016OC
82. Dang AT, Cotton S, Sankaran-Walters S, Li CS, Lee CY, Dandekar S, et al. Evidence of an Increased Pathogenic Footprint in the Lingual Microbiome of Untreated HIV Infected Patients. *BMC Microbiol* (2012) 12:153. doi: 10.1186/1471-2180-12-153
83. Mukherjee PK, Chandra J, Retuerto M, Tatsuoka C, Ghannoum MA, McComsey GA. Dysbiosis in the Oral Bacterial and Fungal Microbiome of HIV-Infected Subjects Is Associated With Clinical and Immunologic Variables of HIV Infection. *PLoS One* (2018) 13(7):e0200285. doi: 10.1371/journal.pone.0200285
84. Annavajhala MK, Khan SD, Sullivan SB, Shah J, Pass L, Kister K, et al. Oral and Gut Microbial Diversity and Immune Regulation in Patients With HIV

on Antiretroviral Therapy. *mSphere* (2020) 5(1):e00798–19. doi: 10.1128/mSphere.00798-19
85. Starr JR, Huang Y, Lee KH, Murphy CM, Moscicki AB, Shiboski CH, et al. Oral Microbiota in Youth With Perinatally Acquired HIV Infection. *Microbiome* (2018) 6(1):100. doi: 10.1186/s40168-018-0484-6
86. Liu G, Saxena D, Chen Z, Norman RG, Phelan JA, Laverty M, et al. HIV Infection Affects Streptococcus Mutans Levels, But Not Genotypes. *J Dent Res* (2012) 91(9):834–40. doi: 10.1177/0022034512454298
87. Li Y, Saxena D, Chen Z, Liu G, Abrams WR, Phelan JA, et al. HIV Infection and Microbial Diversity in Saliva. *J Clin Microbiol* (2014) 52(5):1400–11. doi: 10.1128/JCM.02954-13
88. Lewy T, Hong BY, Weiser B, Burger H, Tremain A, Weinstock G, et al. Oral Microbiome in HIV-Infected Women: Shifts in the Abundance of Pathogenic and Beneficial Bacteria Are Associated With Aging, HIV Load, CD4 Count, and Antiretroviral Therapy. *AIDS Res Hum Retroviruses* (2019) 35(3):276–86. doi: 10.1089/aid.2017.0200
89. Presti RM, Handley SA, Droit L, Ghannoum M, Jacobson M, Shiboski CH, et al. Alterations in the Oral Microbiome in HIV-Infected Participants After Antiretroviral Therapy Administration Are Influenced by Immune Status. *AIDS* (2018) 32(10):1279–87. doi: 10.1097/QAD.0000000000001811
90. Iwai S, Fei M, Huang D, Fong S, Subramanian A, Grieco K, et al. Oral and Airway Microbiota in HIV-Infected Pneumonia Patients. *J Clin Microbiol* (2012) 50(9):2995–3002. doi: 10.1128/JCM.00278-12
91. Coker MO, Mongodin EF, El-Kamary SS, Akhigbe P, Obuekwe O, Omoigberale A, et al. Immune Status, and Not HIV Infection or Exposure, Drives the Development of the Oral Microbiota. *Sci Rep* (2020) 10(1):10830. doi: 10.1038/s41598-020-67487-4
92. Bastard JP, Soulie C, Fellahi S, Haim-Boukobza S, Simon A, Katlama C, et al. Circulating Interleukin-6 Levels Correlate With Residual HIV Viraemia and Markers of Immune Dysfunction in Treatment-Controlled HIV-Infected Patients. *Antivir Ther* (2012) 17(5):915–9. doi: 10.3851/IMP2093
93. Neuhaus J, Jacobs DRJr., Baker JV, Calmy A, Duprez D, La Rosa A, et al. Markers of Inflammation, Coagulation, and Renal Function Are Elevated in Adults With HIV Infection. *J Infect Dis* (2010) 201(12):1788–95. doi: 10.1086/652749
94. Griffen AL, Thompson ZA, Beall CJ, Lilly EA, Granada C, Treas KD, et al. Significant Effect of HIV/HAART on Oral Microbiota Using Multivariate Analysis. *Sci Rep* (2019) 9(1):19946. doi: 10.1038/s41598-019-55703-9
95. Kistler JO, Arirachakaran P, Poovorawan Y, Dahlen G, Wade WG. The Oral Microbiome in Human Immunodeficiency Virus (HIV)-Positive Individuals. *J Med Microbiol* (2015) 64(9):1094–101. doi: 10.1099/jmm.0.000128
96. Goldberg BE, Mongodin EF, Jones CE, Chung M, Fraser CM, Tate A, et al. The Oral Bacterial Communities of Children With Well-Controlled HIV Infection and Without HIV Infection. *PLoS One* (2015) 10(7):e0131615. doi: 10.1371/journal.pone.0131615
97. Fidel PL Jr, Moyes D, Samaranayake L, Hagensee ME. Interplay Between Oral Immunity in HIV and the Microbiome. *Oral Dis* (2020) 26(Suppl 1):59–68. doi: 10.1111/odi.13515
98. Moyes DL, Saxena D, John MD, Malamud D. The Gut and Oral Microbiome in HIV Disease: A Workshop Report. *Oral Dis* (2016) 22(Suppl 1):166–70. doi: 10.1111/odi.12415
99. Saxena D, Li Y, Yang L, Pei Z, Poles M, Abrams WR, et al. Human Microbiome and HIV/AIDS. *Curr HIV/AIDS Rep* (2012) 9(1):44–51. doi: 10.1007/s11904-011-0103-7
100. Navazesh M, Mulligan R, Pogoda J, Greenspan D, Alves M, Phelan J, et al. The Effect of HAART on Salivary Microbiota in the Women's Interagency HIV Study (WIHS). *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* (2005) 100(6):701–8. doi: 10.1016/j.tripleo.2004.10.011
101. Klatt NR, Cheu R, Birse K, Zevin AS, Perner M, Noel-Romas L, et al. Vaginal Bacteria Modify HIV Tenofovir Microbicide Efficacy in African Women. *Science* (2017) 356(6341):938–45. doi: 10.1126/science.aai9383
102. Velloza J, Heffron R. The Vaginal Microbiome and Its Potential to Impact Efficacy of HIV Pre-Exposure Prophylaxis for Women. *Curr HIV/AIDS Rep* (2017) 14(5):153–60. doi: 10.1007/s11904-017-0362-z
103. Imahashi M, Ode H, Kobayashi A, Nemoto M, Matsuda M, Hashiba C, et al. Impact of Long-Term Antiretroviral Therapy on Gut and Oral Microbiotas in HIV-1-Infected Patients. *Sci Rep* (2021) 11(1):960. doi: 10.1038/s41598-020-80247-8
104. Sultan AS, Kong EF, Rizk AM, Jabra-Rizk MA. The Oral Microbiome: A Lesson in Coexistence. *PLoS Pathog* (2018) 14(1):e1006719. doi: 10.1371/journal.ppat.1006719
105. Fidel PLJr., Thompson ZA, Lilly EA, Granada C, Treas K, Dubois KR3rd, et al. Effect of HIV/HAART and Other Clinical Variables on the Oral Mycobiome Using Multivariate Analyses. *mBio* (2021) 12(2):e00294–21. doi: 10.1128/mBio.00294-21
106. Kurita-Ochiai T, Seto S, Suzuki N, Yamamoto M, Otsuka K, Abe K, et al. Butyric Acid Induces Apoptosis in Inflamed Fibroblasts. *J Dent Res* (2008) 87(1):51–5. doi: 10.1177/154405910808700108
107. Magrin GL, Strauss FJ, Benfatti CAM, Maia LC, Gruber R. Effects of Short-Chain Fatty Acids on Human Oral Epithelial Cells and the Potential Impact on Periodontal Disease: A Systematic Review of In Vitro Studies. *Int J Mol Sci* (2020) 21(14):4895. doi: 10.3390/ijms21144895
108. Bipath P, Levay PF, Viljoen M. The Kynurenine Pathway Activities in a Sub-Saharan HIV/AIDS Population. *BMC Infect Dis* (2015) 15:346. doi: 10.1186/s12879-015-1087-5
109. Page EE, Greathead L, Metcalf R, Clark SA, Hart M, Fuchs D, et al. Loss of Th22 Cells Is Associated With Increased Immune Activation and IDO-1 Activity in HIV-1 Infection. *J Acquir Immune Defic Syndr* (2014) 67(3):227–35. doi: 10.1097/QAI.0000000000000294
110. Jenabian MA, El-Far M, Vyboh K, Kema I, Costiniuk CT, Thomas R, et al. Immunosuppressive Tryptophan Catabolism and Gut Mucosal Dysfunction Following Early HIV Infection. *J Infect Dis* (2015) 212(3):355–66. doi: 10.1093/infdis/jiv037
111. Huengsberg M, Winer JB, Gompels M, Round R, Ross J, Shahmanesh M. Serum Kynurenine-to-Tryptophan Ratio Increases With Progressive Disease in HIV-Infected Patients. *Clin Chem* (1998) 44(4):858–62. doi: 10.1093/clinchem/44.4.858
112. Byakwaga H, Boum Y2nd, Huang Y, Muzoora C, Kembabazi A, Weiser SD, et al. The Kynurenine Pathway of Tryptophan Catabolism, CD4+ T-Cell Recovery, and Mortality Among HIV-Infected Ugandans Initiating Antiretroviral Therapy. *J Infect Dis* (2014) 210(3):383–91. doi: 10.1093/infdis/jiu115
113. Favre D, Mold J, Hunt PW, Kanwar B, Loke P, Seu L, et al. Tryptophan Catabolism by Indoleamine 2,3-Dioxygenase 1 Alters the Balance of TH17 to Regulatory T Cells in HIV Disease. *Sci Transl Med* (2010) 2(32):32ra6. doi: 10.1126/scitranslmed.3000632
114. Gaardbo JC, Trosied M, Stiksrud B, Midttun O, Ueland PM, Ullum H, et al. Increased Tryptophan Catabolism Is Associated With Increased Frequency of CD161+Tc17/MAIT Cells and Lower CD4+ T-Cell Count in HIV-1 Infected Patients on cART After 2 Years of Follow-Up. *J Acquir Immune Defic Syndr* (2015) 70(3):228–35. doi: 10.1097/QAI.0000000000000758
115. Nobbs AH, Jenkinson HF, Jakubovics NS. Stick to Your Gums: Mechanisms of Oral Microbial Adherence. *J Dent Res* (2011) 90(11):1271–8. doi: 10.1177/0022034511399096
116. Xiao E, Mattos M, Vieira GHA, Chen S, Correa JD, Wu Y, et al. Diabetes Enhances IL-17 Expression and Alters the Oral Microbiome to Increase Its Pathogenicity. *Cell Host Microbe* (2017) 22(1):120–8.e4. doi: 10.1016/j.chom.2017.06.014
117. Zenobia C, Hajishengallis G. Basic Biology and Role of Interleukin-17 in Immunity and Inflammation. *Periodontol 2000* (2015) 69(1):142–59. doi: 10.1111/prd.12083
118. Mcnabb PC, Tomasi TB. Host Defense-Mechanisms at Mucosal Surfaces. *Annu Rev Microbiol* (1981) 35:477–96. doi: 10.1146/annurev.mi.35.100181.002401
119. Marcotte H, Lavoie MC. Oral Microbial Ecology and the Role of Salivary Immunoglobulin A. *Microbiol Mol Biol Rev* (1998) 62(1):71–109. doi: 10.1128/MMBR.62.1.71-109.1998
120. Nogueira RD, Alves AC, Napimoga MH, Smith DJ, Mattos-Graner RO. Characterization of Salivary Immunoglobulin A Responses in Children Heavily Exposed to the Oral Bacterium Streptococcus Mutans: Influence of Specific Antigen Recognition in Infection. *Infect Immun* (2005) 73(9):5675–84. doi: 10.1128/IAI.73.9.5675-5684.2005
121. Hajishengallis G, Michalek SM. Current Status of a Mucosal Vaccine Against Dental Caries. *Oral Microbiol Immunol* (1999) 14(1):1–20. doi: 10.1034/j.1399-302X.1999.140101.x
122. Skott P, Lucht E, Julander I, Dillner J, Bjorling E. Salivary Siga Response in HIV-1 Infection. *J Acquir Immune Defic Syndr* (1999) 21(2):73–80.

123. Kulkarni V, Ruprecht RM. Mucosal IgA Responses: Damaged in Established HIV Infection-Yet, Effective Weapon Against HIV Transmission. *Front Immunol* (2017) 8:1581. doi: 10.3389/fimmu.2017.01581
124. Honda K, Littman DR. The Microbiota in Adaptive Immune Homeostasis and Disease. *Nature* (2016) 535(7610):75–84. doi: 10.1038/nature18848
125. Sun L, Finnegan CM, Kish-Catalone T, Blumenthal R, Garzino-Demo P, La Terra Maggiore GM, et al. Human Beta-Defensins Suppress Human Immunodeficiency Virus Infection: Potential Role in Mucosal Protection. *J Virol* (2005) 79(22):14318–29. doi: 10.1128/JVI.79.22.14318-14329.2005
126. Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, et al. Beta-Defensins: Linking Innate and Adaptive Immunity Through Dendritic and T Cell CCR6. *Science* (1999) 286(5439):525–8. doi: 10.1126/science.286.5439.525
127. Lafferty MK, Sun L, DeMasi L, Lu W, Garzino-Demo A. CCR6 Ligands Inhibit HIV by Inducing APOBEC3G. *Blood* (2010) 115(8):1564–71. doi: 10.1182/blood-2009-06-226423
128. Lafferty MK, Sun L, Christensen-Quick A, Lu W, Garzino-Demo A. Human Beta Defensin 2 Selectively Inhibits HIV-1 in Highly Permissive CCR6(+) CD4(+) T Cells. *Viruses* (2017) 9(5):111. doi: 10.3390/v9050111
129. Yiu JH, Dorweiler B, Woo CW. Interaction Between Gut Microbiota and Toll-Like Receptor: From Immunity to Metabolism. *J Mol Med (Berl)* (2017) 95(1):13–20. doi: 10.1007/s00109-016-1474-4
130. Huan Y, Kong Q, Mou H, Yi H. Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple Fields. *Front Microbiol* (2020) 11:582779. doi: 10.3389/fmicb.2020.582779
131. Brandtzaeg P. Secretory IgA: Designed for Anti-Microbial Defense. *Front Immunol* (2013) 4:222. doi: 10.3389/fimmu.2013.00222
132. Tanzer JM, Fisher J, Freedman ML. Preemption of Streptococcus Mutans 10449S Colonization by Its Mutant 805. *Infect Immun* (1982) 35(1):138–42. doi: 10.1128/iai.35.1.138-142.1982
133. Hillman JD, Mo J, McDonell E, Cvitkovitch D, Hillman CH. Modification of an Effector Strain for Replacement Therapy of Dental Caries to Enable Clinical Safety Trials. *J Appl Microbiol* (2007) 102(5):1209–19. doi: 10.1111/j.1365-2672.2007.03316.x
134. Gruner D, Paris S, Schwendicke F. Probiotics for Managing Caries and Periodontitis: Systematic Review and Meta-Analysis. *J Dent* (2016) 48:16–25. doi: 10.1016/j.jdent.2016.03.002
135. Burne RA. Getting to Know "The Known Unknowns": Heterogeneity in the Oral Microbiome. *Adv Dent Res* (2018) 29(1):66–70. doi: 10.1177/0022034517735293
136. Coker MO, Madan JC. Chapter 3 - The Microbiome and Immune System Development. In: Claud EC, editor. *The Developing Microbiome*. Academic Press (2020). p. 43–66.

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