Chapter 2

‘Talk to your gut’: the oral-gut microbiome axis and its immunomodulatory role in the etiology of rheumatoid arthritis

Marines du Teil Espina*, Giorgio Gabarrini*, Hermie J.M. Harmsen, Johanna Westra, Arie Jan van Winkelhoff, and Jan Maarten van Dijl

*These authors contributed equally

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Abstract

Microbial communities inhabiting the human body, collectively called the microbiome, are critical modulators of immunity. This notion is underpinned by associations between changes in the microbiome and particular autoimmune disorders. Specifically, in rheumatoid arthritis, one of the most frequently occurring autoimmune disorders worldwide, changes in the oral and gut microbiomes have been implicated in the loss of tolerance against self-antigens and in increased inflammatory events promoting the damage of joints. In the present review, we highlight recently gained insights in the roles of microbes in the etiology of rheumatoid arthritis. In addition, we address important immunomodulatory processes, including biofilm formation and neutrophil function, which have been implicated in host-microbe interactions relevant for rheumatoid arthritis. Lastly, we present recent advances in the development and evaluation of emerging microbiome-based therapeutic approaches. Altogether, we conclude that the key to uncovering the etiopathogenesis of rheumatoid arthritis will lie in the immunomodulatory functions of the oral and gut microbiomes.

Introduction

The many trillions of microbes we harbor in our bodies are not pure spectators. Indeed, they play a fundamental role in shaping our immune system and metabolism as has become increasingly evident in recent years[1-5]. These microbes, which altogether constitute our microbiome, are located in the gastrointestinal tract, the nose, the oral cavity, the skin, the vagina, and, to a lesser extent, the lungs[1, 3]. Interestingly, compositional changes of the microbiome, altogether categorized as dysbiosis[1], have been associated with a broad range of diseases including metabolic and autoimmune disorders[1, 3, 5]. Since then, efforts have been made to define a "healthy microbiome", but only as of late, with the use of sophisticated sequencing technologies and computational methods for data analysis, bountiful progress has been made in this field[6, 7]. One important example of this progress is the Human Microbiome Project[8-10], implemented by the US National Institutes of Health. The large-scale high-throughput analyses performed in this project yielded over 350 papers providing important clues on how the microbiome and its expressed genes play a role in health and disease[3]. Dysbiotic conditions have therefore been the subject of critical studies, especially to uncover factors leading to this unbalance of the complex *status quo* in which microbial communities interact within and with the human body. Factors altering microbial homeostasis include the use of antibiotics and other drugs, changes in diet patterns, elimination of constitutive nematodes, the introduction of a new microbial actor, and ageing[1, 2, 4, 5, 11-13].

Intriguingly, despite the associations between microbiome and autoimmunity, the tissue targeted by autoimmune disorders is often not the same tissue where the microbiome is thought to exert its pathogenic role[14, 15]. This is clearly exemplified by rheumatoid arthritis (RA), one of the most prevalent autoimmune diseases, affecting approximately 1% of the human population[16]. RA thus contributes significantly to the global morbidity and mortality and, according to the allegations of its increasingly higher incidence among the elderly population[17, 18], it is a major threat to healthy ageing[19, 20]. RA is characterized by a persistent synovial inflammation, which ultimately results in articular cartilage and bone damage[21]. Recent models have implicated the involvement of loss of tolerance toward citrullinated proteins in RA development[22-24]. Citrullination is a post-translational protein modification involving the transformation of a positively charged arginine residue into a neutral citrulline residue[22]. This reaction is catalyzed by peptidylarginine deiminase (PAD) enzymes, which are

extremely well conserved among mammals[25]. Of note, human PAD enzymes regulate, in a variety of cells and tissues, important processes such as apoptosis, inflammatory immune responses, and the formation of rigid structures like skin or myelin sheaths[26-28]. Consistent with RA etiological models, in the majority of predisposed subjects, the presence of citrullinated proteins gives rise to specific autoantibodies called anti-citrullinated protein antibodies (ACPAs)[23, 29, 30]. Remarkably, ACPAs have a specificity of 95% and are 68% sensitive for RA[31, 32]. These auto-antibodies can be detected years before the appearance of clinical symptoms[33]. Moreover, their serum levels strongly correlate with disease severity, hinting at a possible role in the progression of the disease[34].

The etiology of RA is still not fully understood but, among its potential causes, certain genetic factors were shown to strongly correlate with the disease. Particularly, the major histocompatibility complex (HLA)-DRB1 locus is one of the most well-established genetic risk factors associated with RA and ACPAs[21]. Specifically, alleles coding for a five amino acid sequence called shared epitope, which is present in the HLA-DRB1 region, are carried by 80% of $ACPA^+$ RA patients[35] and correlate with disease activity and mortality[36, 37] (Fig. 1). The shared epitope appears to favor the binding of citrulline-containing peptides during HLA presentation when compared to their non-citrullinated counterparts, although this hypothesis seems to be applicable only to certain shared epitope alleles such as HLA-DRB1*04:01, *04:04 and *04:05[38, 39]. Nevertheless, it appears that the genetic component is only one of the many RA-contributing factors. Specifically, environmental ones have always attracted great attention for multiple reasons. In particular, it is noteworthy that the genetic component is not sufficient to explain the recent increase in RA prevalence among the population[40]. An additional, more intuitive, reason is that not every individual carrying the alleles implicated in RA susceptibility develops RA[41]. Important clues for the identification of environmental triggers of RA were provided in the beginning of the 20^{th} century, when treatment of periodontal infections were proven to ameliorate symptoms of patients with rheumatoid arthritis[42]. Since then, it has become increasingly more evident that oral health and especially the oral microbiome significantly influence the progression of RA[16, 43]. Studies consistent with this line of thought revealed another, less apparent, actor playing a role in the pathogenesis of RA: the gut microbiome[44] (Fig. 1).

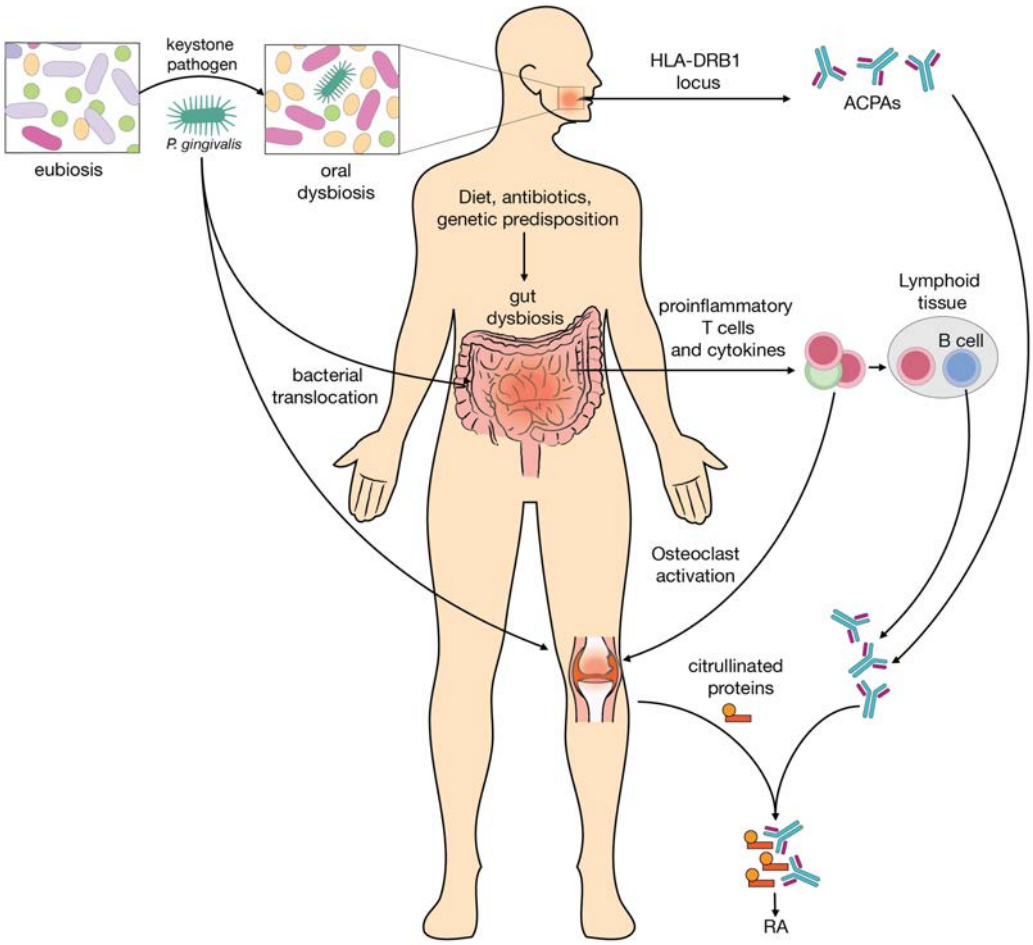

Figure 1. *Model of the influence of oral and gut microbiomes on RA.* Dysbiosis of the oral microbiome is mediated by the keystone pathogen *Porphyromonas gingivalis*. This bacterium, through direct and indirect increase of the citrullination burden, may mediate ACPA production in the oral cavity. Additionally, *P. gingivalis* may be involved in gut dysbiosis due to its purported translocation to the gut. Gut dysbiosis, in turn, leads to the production of Th1, Th17 cells, and pro inflammatory cytokines, all of which can enter the blood stream and localize in lymphoid tissues. In here, they can activate autoreactive B cells, which produce ACPAs. ACPAs produced both in the oral cavity and in the lymphoid tissues can migrate to the joints and potentially contribute to RA onset. Two other related sources of damage in the joints are IL-17-induced osteoclastogenesis and aberrant concentration of citrullinated proteins. Osteoclastogenesis can be directly mediated by IL-17 produced by Th17 cells, which can migrate from the gut to the joints. Moreover, in case of an inflammatory status of the joints due to the potential translocation of *P. gingivalis* components, high levels of citrullinated peptides are produced. When these peptides are targeted by ACPAs in individuals with a genetic predisposition, RA can develop.

Indeed, Zhang *et al.* recently analyzed the microbiome composition of fecal, dental, and salivary samples of RA patients, showing that both the oral and gut microbiomes were dysbiotic compared to the ones of healthy individuals[45]. Strikingly, the dysbiotic characteristics were shown to be partially resolved after RA treatment, which implied an interplay between RA and the oral-gut axis[45]. Understanding the role of the microbiome in RA

is therefore essential to fully understand the etiopathological landscape of RA. Additionally, this insight might also be useful in understanding similar, related, autoimmune diseases such as systemic lupus erythematosus (SLE)[46]. In this review, we discuss the most relevant findings on how the interplay of both the oral and gut microbiomes with the host mediate RA onset, focusing on recently proposed factors such as biofilms and neutrophil function. Lastly, we will address how this information could eventually lead to the identification of potentially druggable targets for a microbiome-based therapeutic management of RA and other autoimmune diseases.

Oral microbiome, periodontitis and RA

Oral health has been clinically associated with autoimmune diseases in a number of epidemiological studies[29, 47-51] (Tables 1 and 2). An important example of this is the correlation between RA and periodontitis, which is a chronic inflammatory disorder affecting the periodontium, the tissue supporting the teeth[47]. Periodontitis is a major cause of tooth-loss and one of the most widespread diseases in the world, with an incidence of roughly 11% in the human population[16], although the disease affects between 10 to 57% of different populations worldwide, depending on severity, socio-economic status, and oral hygiene[52]. As mentioned, a recent cause of concern for this disease is its long-known correlation with RA[29, 48]. It has been reported, in fact, that periodontitis patients have twice the chance of contracting rheumatoid arthritis and RA patients are twice as likely to become edentulous[29, 47, 53-55].

Table 1. *List of oral bacteria associated with RA pathogenesis, and related mechanisms.*

Bacteria implicated	Mechanistic insight linking the oral microbiome to RA	Methodology	Study findings	Study
P. gingivalis	-	Correlation of antibody responses against *P. gingivalis* and/or *P. gingivalis* proteins, determined by ELISA, with RA.	Anti-*P. gingivalis* levels higher in patients with RA vs non-RA controls.	(Tolo *et al.* 1990)
			Significantly elevated Anti-RgpB antibodies in PD vs non-PD, RA vs non-RA and ACPA$^+$ RA vs ACPA$^-$ RA groups. Significant correlation between anti-RgpB antibodies and RA even more than with smoking.	(Kharlamova *et al.* 2016)
			Anti-*P. gingivalis* levels higher in patients with RA vs non-RA, and in ACPA$^+$ RA vs ACPA$^-$ RA groups.	(Hitchon *et al.* 2010)
			Significant association between anti-PPAD antibodies and ACPAs.	(Shimada *et al.* 2016)

		Anti-PPAD response elevated in RA vs non-RA and PD vs non-PD groups.	(Quirke *et al*. 2014)
		Anti-PPAD response does not correlate with ACPAs and disease activity in RA. Anti-PPAD antibody levels are significantly lower in PD^+ RA patients compared PD^- RA.	(Konig *et al.* 2014)
Molecular mimicry	Cross reactivity of human citrullinated proteins with bacterial citrullinated proteins determined by ELISA, immunoblotting and/or mass spectrometry.	Antibodies against an immunodominant epitope in citrullinated human alpha enolase cross-reacted with citrullinated *P. gingivalis* enolase.	(Lundberg *et al*. 2008)
		ACPAs cross-reacted with outer membrane antigens and citrullinated *P. gingivalis* enolase.	(Li *et al*. 2016)
Induction of Th17 responses	Th17 representation in *ex vivo* periodontal tissues of PD patients. *In vitro* cytokine production by cells exposed to *P. gingivalis.*	Large number of Th17 and enhanced IL-17 production in PD tissues compared to controls. Production of Th17 related cytokines induced by *P. gingivalis,* a mechanism favored by *P. gingivalis* proteases.	(Moutsopoulos *et al.* 2012)
	Induction of periodontitis in mice and subsequent Th17 detection in selected tissues.	Accumulation of Th17 in the oral mucosa and draining lymph nodes induced by oral microbiota.	(Tsukasaki *et al*. 2018)
	Induction of periodontitis in experimental arthritis mice model with *in vitro* exposure of lymph node cells to both bacteria.	Periodontitis induced by both bacteria significantly aggravated arthritis, which was characterized by predominant Th17 cell responses in draining lymph nodes. Th17 induction by *P. gingivalis* and *P. nigrescens* was strongly dependent on the activation of antigen presenting cells *via* TLR2 and was enhanced by the production of IL-1 by these cells.	(de Aquino *et al*. 2014)
PPAD citrullination	Infection with PPAD- proficient or deficient *P. gingivalis* of an experimental arthritis-induced mice model.	*P. gingivalis* infection aggravated arthritic symptoms in a PPAD-mediated manner. Significantly higher levels of autoantibodies and citrullinated proteins observed in mice infected with PPAD-proficient *P. gingivalis*.	(Maresz *et al.* 2013)
		Increased arthritic symptoms and ACPA levels observed in mice infected with PPAD-proficient *P. gingivalis*.	(Gully *et al.* 2014)
Microbial translocation	Oral infection with "red complex" bacteria prior to induction of arthritis in mice. Detection of bacteria in remote tissues by PCR and FISH.	Presence of periodontal bacteria in synovial joints correlated with arthritis severity. Presence of *P. gingivalis* in the perinuclear area of cells in joint tissues.	(Chukkapalli *et al*. 2016)

		Detection of bacterial DNA by PCR in subgingival dental plaque, synovial fluid, and serum of RA patients with PD.	*P. gingivalis* and *P. intermedia* were the species more often found in the subgingival dental plaque and synovial fluid of RA patients with PD.	(Martinez-Martinez *et al.* 2009)
		Synovial fluid and tissues of RA patients were examined for the presence of *P. gingivalis* DNA determined by PCR.	Higher levels of *P. gingivalis* DNA found in synovial tissues of RA patients compared to control.	(Totaro *et al*. 2013)
	Modulation of the gut microbiome	Oral infection with *P. gingivalis* or *P. intermedia* with subsequent arthritis induction. Determination of changes in gut immune system and gut microbiome composition.	*P. gingivalis* significantly aggravated arthritis, increased Th17 proportions and IL-17 production, and changed the gut microbiome composition.	(Sato *et al.* 2017)
A.actinom-ycetemcomitans (*Aa*)	Hypercitrulli-nation induced by LtxA of *Aa*	Mass spectrometry of gingival crevicular fluid. *In vitro* challenge of human neutrophils. Correlation between anti-*Aa* responses and RA and ACPAs, by ELISA.	RA joint citrullinome mirrors the one in the PD oral environment. Hypercitrullination in human neutrophils induced by the pore-forming toxin LtxA of *Aa*. Neutrophil challenge with LtxA generated citrullinated RA autoantigens. Anti-LtxA and anti-*Aa* responses correlated with ACPAs and RA.	(Konig *et al.* 2016)
Prevotella intermedia	-	Mass spectrometry of gingival crevicular fluid. ELISA of selected citrullinated peptides performed on RA serum.	Antibody responses against a novel citrullinated peptide cCK13-1 were elevated in RA patients. Anti–cCK13-1 and anti-cTNC5 were associated with anti-*P. intermedia* responses.	(Schwenzer *et al.* 2017)

Table 2. *List of microbiomes associated with RA pathogenesis, and related mechanisms.*

Microbiome implicated	Methodology	Study findings	Study
Oral	16S rRNA gene sequencing of subgingival plaque samples	Higher abundance of Gram-negative inflamophilic bacteria, including *Prevotella* spp.and *Leptotrichia* spp. in RA patients, compared to non-RA controls. *Cryptobacterium curtum* as a discriminant between RA and non-RA patients	(Lopez-Oliva *et al.* 2018)

Oral	16S rRNA gene sequencing of subgingival plaque samples; ELISA	Lower abundance of *A. germinatus*, *Haemophilus* spp., *Aggregatibacter* spp., *Porphyromonas* spp., *Prevotella* spp., *Treponema* spp. in RA patients compared to OA controls.	(Mikuls *et al.* 2018)
Oral	Pyrosequencing of subgingival plaque samples; ELISA	Higher abundance of *Prevotella* spp. and *Leptotrichia* spp. in new-onset RA patients. ACPA correlated with *A. germinatus*. Similar exposure to *P. gingivalis* among groups.	(Scher *et al.* 2012)
Oral and gut	Metagenomic shotgun sequencing of fecal, dental and salivary samples	Lower abundance of *Haemophilus* spp. and higher abundance of *Lactobacillus salivarius* in RA patients vs non-RA controls.	(Zhang *et al.* 2015)

Additionally, treatment of periodontitis has been shown to ameliorate symptoms of rheumatoid arthritis and *vice versa*[56-59], and the citrullinome of periodontopathic conditions mirrors the one of the arthritic inflamed joint[60]. However, the molecular mechanism behind this association has not yet been elucidated. Nevertheless, strong evidence suggests that RA autoimmunity is triggered or enhanced by specific oral bacteria that are causatives of periodontal disease[27, 49, 60-62]. The Gram-negative bacterium *Porphyromonas gingivalis* is the main suspect in the association between periodontitis and RA[19]. This was firstly due to the fact that antibody responses against *P. gingivalis* and specific *P. gingivalis* virulence factors appeared to correlate with RA severity and ACPA levels[63-65], even more strongly than with smoking, a well-known RA risk factor[65]. Secondly, in more recent times, a peculiar *P. gingivalis* enzyme has been hailed as the lynchpin of the link between periodontitis and RA[66]. This protein is the PAD enzyme of *P. gingivalis* (PPAD), the only thus far reported PAD enzyme produced by a human pathogen[25, 67, 68]. Antibodies against PPAD, in fact, have been shown to correlate with RA in several studies[23, 69]. Albeit contradicting observations have been made[45, 70], PPAD involvement in RA development was implied by experimental studies in RA murine models[62, 71]. In these studies, either genetically engineered PPAD-deficient *P. gingivalis* mutants or the wild-type strains were used to infect mice in which arthritis was experimentally induced. A higher autoantibody production as well as higher joint damage were observed in mice infected with the wild-type strain compared to the ones infected with PPAD-deficient mutants, suggesting a role for PPAD in the exacerbation of RA. This bacterial enzyme is evolutionary unrelated to mammalian PADs, but it nonetheless shares with this group of eukaryotic enzymes the catalytic function[34]. Of note, PPAD is purported to play a role in RA etiology with two potential mechanisms. The first one requires the proteolytic activity of a specific class of highly efficient proteases secreted by *P. gingivalis*, named

arginine-gingipains, which were shown to be necessary for α-enolase citrullination[27]. *In vitro* experiments showed that cleavage of host proteins by gingipains, in fact, exposes carboxyl-terminal arginine residues, which are the preferential targets of PPAD[27, 72]. This unique mode of citrullination of cleaved peptides may be the basis of the generation of so-called neo-epitopes at sites where PPAD activity has been suggested, such as the sites of infection or even distant periodontal tissues[73]. Neo-epitopes are epitopes to which immune tolerance has not yet been developed, consequently triggering an autoimmune response[27] (Fig. 2). The second mechanism involves molecular mimicry (Fig. 2). It has been shown, in fact, that autoantibodies directed against the immunodominant epitope of human citrullinated α-enolase cross-react with *P. gingivalis* citrullinated α-enolase[74]. These observations were further confirmed by Li *et al.*, who additionally identified six *P. gingivalis* citrullinated peptides recognized by RA-derived ACPAs[75]. Besides the hypotheses proposing a causative relationship between PPAD production and RA autoimmunity, however, other oral microbiome-driven mechanisms mediating loss of tolerance against citrullinated proteins have been proposed. The first is enhanced human PAD-mediated citrullination[62]. Inflammatory processes that can be triggered by microbial events, in fact, have been known to involve PAD-mediated citrullination. In the case of chronic inflammations, such as periodontitis, continuous PAD activation might lead to an enhanced citrullination burden and, potentially, autoimmunity[76, 77] (Fig. 2). Dysbiosis is therefore considered to be a critical driver for the perpetuation of inflammatory statuses and break in tolerance against citrullinated proteins[78, 79].

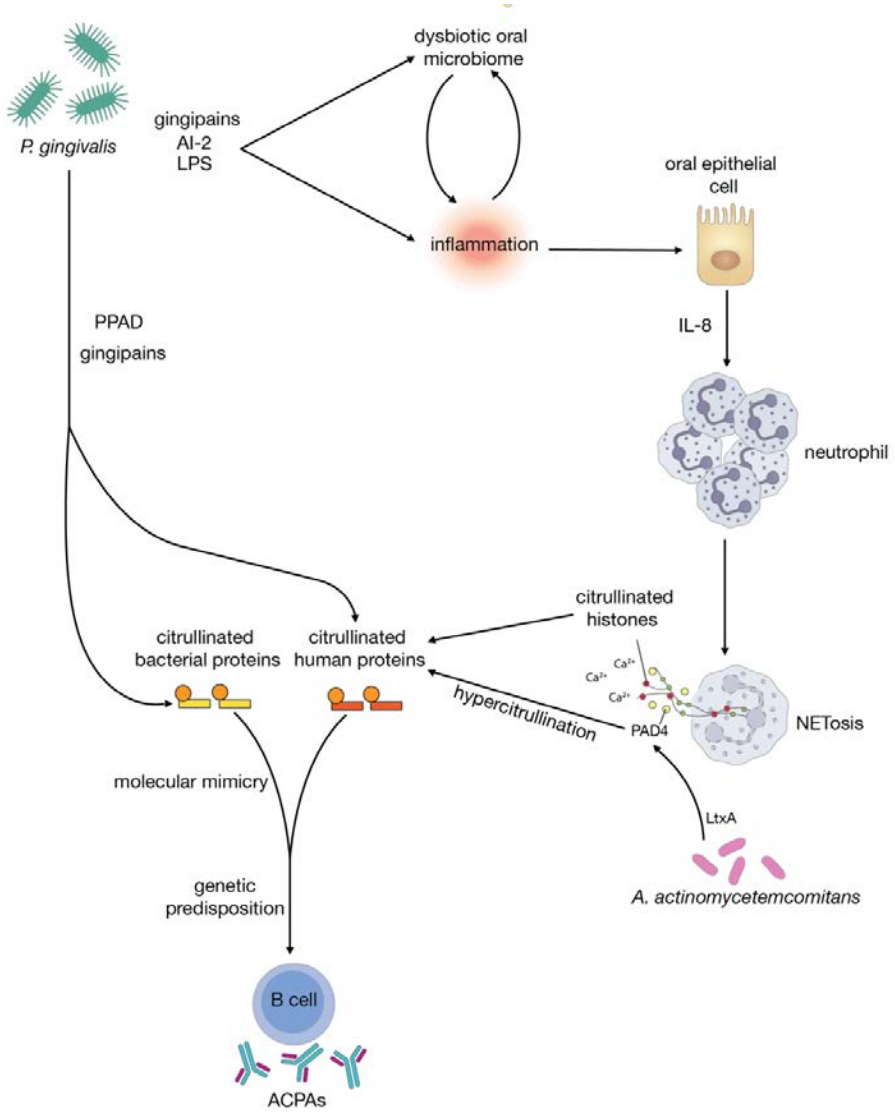

Figure 2. *Oral microbiome-driven mechanisms that potentially contribute to RA.* Members of the oral microbiome, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, are actors in the complex interplay of mechanisms leading to the production of ACPAs. *P. gingivalis* can mediate the creation of citrullinated proteins through secretion of gingipains and PPAD. In turn, bacterial citrullinated proteins might elicit ACPA formation in genetically predisposed subjects *via* molecular mimicry. Additionally, *P. gingivalis* can indirectly contribute to citrullination by mediating proinflammatory events. Indeed, through secretion of quorum sensing molecules, such as AI-2, and through gingipains and lipopolysaccharide, *P. gingivalis* is able to promote inflammation and dysbiosis. Dysbiosis in turn triggers inflammation, which is favorable for the persistence of dysbiotic bacteria, creating a positive feedback loop between the two phenomena. In this scenario, epithelial cells secrete the proinflammatory cytokine IL-8, which recruits and activates neutrophils, promoting enhanced NETosis. Consequently, intracellular citrullinated antigens, such as citrullinated histones, are exposed and released in the extracellular milieu. This release of citrullinated epitopes might be an additional driver for the rise of ACPAs in genetically predisposed individuals. Moreover, the human PAD enzyme PAD4 is simultaneously released in the extracellular environment upon the neutrophil lytic event. The calcium-rich conditions of the extracellular milieu might lead PAD4 to hypercitrullinate human proteins, thus increasing the overall citrullination burden and potentially resulting in ACPA formation. *A. actinomycetemcomitans* may also break the tolerance against citrullinated antigens, driving ACPA production by B cells in genetically predisposed individuals with its enzyme LtxA. This protein, in fact, is responsible for permeabilizing the neutrophil membrane, allowing the release of PAD4.

Interestingly, *P. gingivalis*, albeit underrepresented in the periodontal oral microbiome, appears to be capable of causing inflammatory responses by orchestrating oral dysbiosis[80, 81]. This peculiar feat, which placed *P. gingivalis* in the limelight as a "keystone pathogen", creates a suitable environment for dysbiotic bacteria to persist, aggravating the loop between oral dysbiosis and inflammation[80] (Fig. 2).

Besides a direct or indirect modulation of citrullination, the oral microbiome influences other processes, mainly involving the T cell-mediated adaptive immunity, that have been correlated with chronic inflammation and bone damage in the RA joints[82, 83]. Specifically, T helper 17 (Th17) cells, a subset of $CD4^+$ T cells normally produced against bacterial or fungal infections, have been associated with joint damage *via* mechanisms such as overproduction of the proinflammatory cytokines IL-17A, IL-17F, and IL-22, cross-reactivity with joint-derived antigens, or migration to the joints, where increased osteoclast activation mediates bone resorption[84-87]. These pathological Th17 cells can be produced in the oral cavity in response to certain periodontal pathogens[88-90]. Accordingly, Th17 cells and Th17-related cytokines are often observed in *ex vivo* gingival tissue samples of periodontitis patients[88]. Additionally, a recent study using a periodontitis mouse model was characterized by accumulation of, among CD4+ T cell subsets, only Th17 cells. This accumulation was reverted after administration of antibiotics, corroborating the hypothesized role of the oral microbiome in the production of Th17 cells and their ensuing responses[89]. Accordingly, *P. gingivalis* was shown to specifically induce the production of Th17-related cytokines *in vitro,* a mechanism that involved gingipain degradation of specific cytokine mediators that favored Th17 responses[88]. Moreover, it was later confirmed in collagen-induced arthritis (CIA) mice, that induction of periodontitis by *P. gingivalis* and another Gram-negative bacterium, *Prevotella nigrescens*, resulted in increased presence of Th17 cells in lymph nodes draining arthritic joints, and in aggravation of arthritic symptoms[90]. The mechanisms by which Th17 responses are enhanced by these two oral pathogens involved IL-1 activity and the activation of antigen-presenting cells via Toll Like Receptor 2[90]. Additional, less explored, mechanisms underlying the interplay of *P. gingivalis* and RA etiology are further detailed in particular dedicated sections of this review.

In recent years, studies investigating RA pathogenesis have implicated other periodontal pathogens aside from *P. gingivalis* in this disease. Schwenzer *et al.* demonstrated that the serological response against *Prevotella intermedia* in RA patients was associated with a novel ACPA directed against cCK13-1, a newly discovered citrullinated peptide of cytokeratin 13,

found in the periodontium[91]. Interestingly, unlike other ACPAs, this autoantibody did not correlate with a serological response against *P. gingivalis*, suggesting that ACPAs with different specificities might arise from responses to different oral periodontal pathogens[91].

Another study, has recently implicated the Gram-negative bacterium *Aggregatibacter actinomycetemcomitans* in the etiology of RA through the enhancement of citrullination[60]. The mechanism behind this purported association appears to depend on the pore-forming leukotoxin of *A. actinomycetemcomitans*, LtxA. Upon a lytic stimulus from this toxin, destruction of the neutrophil membrane occurs, thus releasing human PADs and leading to hypercitrullination[80] (Fig. 2). A correlation between LtxA and RA was further demonstrated, as anti-LtxA antibodies were associated with ACPA serum titers in RA patients. The biomolecular rationale behind this mechanism is further explained in the "Neutrophils and RA pathogenesis" section below.

Aside from the aforementioned studies, which have investigated the involvement of specific oral species in RA etiopathogenesis, efforts have been made to analyze the oral microbiome composition in RA patients. Scher *et al.* 2012 analyzed the microbial composition of rheumatoid arthritis and control patients with and without periodontitis. New-onset RA patients (NORA), chronic RA (CRA) patients, and healthy control volunteers were included in this study, in order to pinpoint specific bacteria that are associated with different stages of RA progression. Among all groups analyzed, NORA patients exhibited high incidence of advanced periodontal disease. Intriguingly, the microbial richness and composition did not show a significant variation among all groups with a similar periodontitis status[92]. However, two taxa of Gram-negative bacteria were exclusively found in NORA patients irrespective of periodontal disease, namely *Prevotella* spp. and *Leptotrichia* spp.[92]. Moreover, ACPA levels were positively associated with the presence and abundance of yet another Gram-negative bacterium, *Anaeroglobus geminatus*, indicating a possible role of this bacterium in RA initiation. An unexpected finding was that presence and abundance of *P. gingivalis* was not positively associated with RA or with ACPA serum titers, but only with periodontitis severity[92].

Zhang *et al.* 2015, on the other hand, analyzed fecal, dental and salivary samples of RA patients observing a dysbiotic gut and oral microbiota compared to healthy individuals. Particular attention was given to Gram-negative bacterial *Haemophilus* species, which were underrepresented in the oral and gut compartments of RA patients and which negatively correlated with autoantibodies related to RA[45]. In contrast, the Gram-positive *Lactobacillus*

salivarus was overrepresented in all body sites tested of RA patients and positively correlated with disease activity[45]. Lopez-Oliva *et al.* also analyzed the oral microbiome composition in periodontally healthy individuals with or without RA. Similarly to Zhang *et al.*, the study showed that the microbiome of RA patients is enriched for certain Gram-negative species with proinflammatory capacity including *Prevotella* spp. and, similarly to Scher *et al.*, *Leptotrichia* spp.*,* suggesting a possible role for these two bacteria in the initiation of RA[92, 93]. Additionally, the Gram-positive *Cryptobacterium curtum* was identified as the predominant species in the microbiome of RA patients[93]. This is of interest particularly due to *C. curtum*'s capability of citrullinating free arginine through the arginine deiminase pathway, albeit ACPAs target citrullinated proteins and not free citrulline.

Another recent study[94] investigated the subgingival microbiome of RA patients using as control the microbiome of osteoarthritis (OA) patients, in order to pinpoint specific correlations with the autoimmune side of rheumatoid arthritis. Interestingly, after taking the periodontal status into account, no robust microbial fingerprint was found for RA when compared to OA[94]. Remarkably, in contrast with previous studies, no correlation was observed between serum ACPA levels and abundance of bacteria that have been associated with RA, such as *P. gingivalis*, *A. germinatus*, *Haemophilus,* or *Aggregatibacter*[94]. Additionally, an under-representation of *Peptostreptococcus*, *Porphyromonas*, *Prevotella* and *Treponema species* was observed in RA patients with periodontitis compared to OA patients with periodontitis. Of note, early RA patients also presented an under-representation of *Prevotella* and *Porphyromona*s species in the microbiome of their lung, which has recently emerged as another important extra-articular site where RA autoimmunity may develop[95]. It must be noted that all the aforementioned metagenomic studies are correlational and therefore do not necessarily imply involvement of specific bacteria in the causation of a disease. Moreover, abundance of a microbe does not always correlate with the serological host response[49], a factor known to have implications in ACPA formation and RA[49, 75, 91]. The results of these studies, however, albeit not giving insights into the mechanisms behind the pathogenesis of RA, might lead to advancements in diagnosis and prognosis of this disease.

Oral biofilms and their role in inflammatory responses

One of the factors leading to chronic inflammation in periodontitis is the dental biofilm, which consists of highly complex, organized, and dynamic microbial communities that acquire in this way resistance to environmental stresses, including antibiotics[96-98] (Fig. 3). For this reason, biofilms have been studied thoroughly by the medical community. However, direct correlations between biofilms and autoimmune disorders have not yet been examined extensively. Nevertheless, in recent years, studies showed the immuno-modulating properties of specific biofilm mediators responsible for biofilm formation. Some of such mediators are called autoinducers, the most conserved signaling molecules that allow "communication" among bacteria in an interconnecting process known as quorum sensing[96]. Specifically, the quorum sensing molecule autoinducer 2 (AI-2), which can be secreted and sensed by both Gram-positive and Gram-negative bacteria, has been shown to mediate the virulence and biofilm formation of periodontal pathogens[99]. Moreover, the involvement of AI-2 in inflammation processes was recently demonstrated by Zargar *et al.,* who analyzed the transcriptome of human intestinal epithelial cells (IECs) when exposed to proteins secreted by two strains of non-pathogenic *Escherichia coli* that differ mainly in their production of AI-2. The differential inflammatory response of the IECs prompted the authors to study the specific role of AI-2 by stimulating these cells with synthetic AI-2[100]. Their results suggest that IECs are able to alter the transcription of immune mediators, such as the neutrophil-recruiting interleukin 8 (IL-8), when faced with quorum sensing molecules.

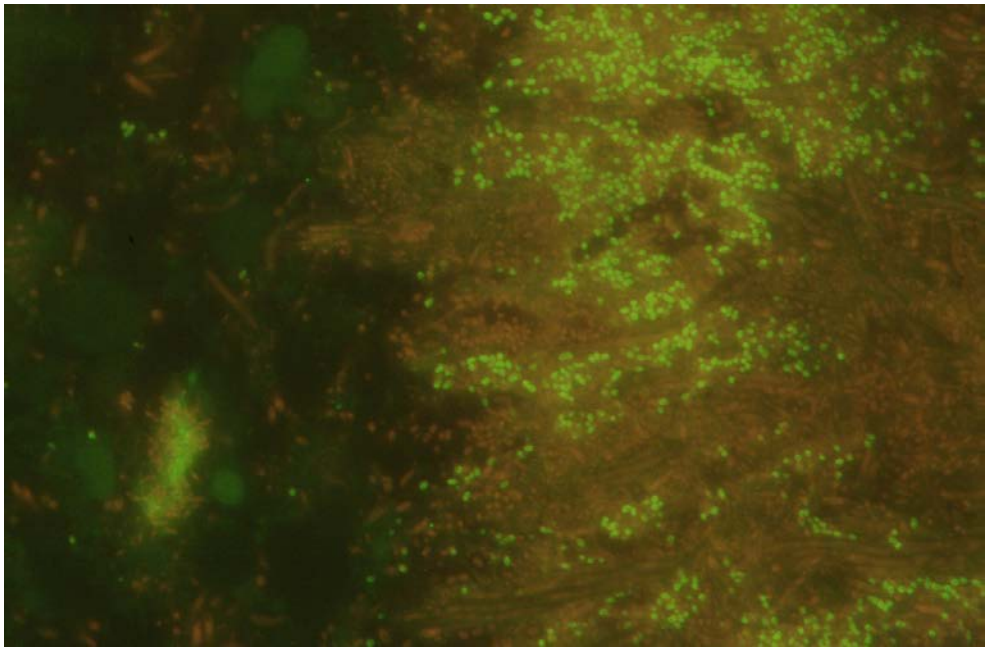

Figure 3. *Visualization of Porphyromonas gingivalis in the oral biofilm*. Fluorescent in situ hybridization (FISH) analysis of an *in vivo* grown oral biofilm shows microcolonies of *P. gingivalis*, stained in green, that are localized in the top layer of the biofilm. *P. gingivalis* was visualized using the FISH probe PG477FITC, and additional eubacteria in the biofilm (red background staining) were visualized with the FISH probe EUB338Cy3 as described in Zijnge *et al*. (2010)[92].

Moreover, in the case of RA and its alleged relationship with periodontitis, AI-2 molecules have been demonstrated to mediate oral biofilm formation[98]. Furthermore, AI-2 molecules were found to be expressed by periodontal pathogens, such as *P. gingivalis*[101, 102], belonging to one of the nine taxa composing the main core of dental biofilms, termed hedgehog structure[103]. Of note, this bacterium has been found capable of inducing secretion of IL-8 in oral epithelial cells[104]. This observation is apparently supported by the finding that addition to oral bacteria of the "red complex", a group of bacteria comprising *P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, can increase the IL-8 production in oral epithelial cells[100, 105]. Taken together, these results suggest that quorum sensing signaling molecules released by *P. gingivalis* might lead to an inflammatory response in the oral cavity, as schematically represented in Figure 2. As biofilms have been proven to represent inflammation-causing etiological factors of periodontitis, studies delving deeper into this topic might also help widening our understanding of the periodontal field and potentially inflammation-driven autoimmunity.

Neutrophils and RA pathogenesis

Another suggested oral microbiome-mediated mechanism potentially contributing to autoimmunity involves neutrophils. Neutrophils act as a first line of defense in periodontal diseases and are important regulators of both innate and adaptive immunity. Aberrant neutrophil functions have recently emerged as actors in the initiation and pathogenesis of autoimmune diseases, such as SLE and RA[106, 107]. In SLE, neutrophils exhibit impaired phagocytosis, have a tendency to form aggregates, display an elevated apoptotic behavior and an increased activation state mediated by nucleosomes[108, 109]. Similarly, in RA, an increased recruitment and activation of neutrophils in synovial fluid can be observed in the early stages of the disease[110]. A similar neutrophil phenotype is present in periodontitis where it has been suggested that neutrophils are key players in the initiation and perpetuation of the inflammation of gingival tissue[111]. Interestingly, one important neutrophilic mechanism, which was recently strongly correlated with autoimmunity in SLE and RA, is the production of neutrophil extracellular traps (NETs), a process also known as NETosis. The NETosis mechanism involves the release, after the lysis of a neutrophil, of decondensed chromatin with, bound to it, a variety of proteins such as histones and antimicrobial peptides, forming traps for targeted microorganisms[107]. One essential step in NETosis is the citrullination of nuclear proteins such as histones (specifically H2A, H3, and H4) by the PAD4 enzyme, which is abundantly expressed in neutrophils[112] (Fig. 4). Notably, in healthy individuals, NETs are cleared after they have performed their extracellular killing function. The clearance of NETs is known to be performed by extracellular DNAse I and by macrophages, which can engulf and digest NETs[113, 114]. This function might be fundamental, considering that release into the extracellular environment of NET components, such as citrullinated proteins and DNA, might create a potential new source of autoantigens in genetically susceptible hosts, thereby potentially contributing to autoimmune diseases[115] (Fig. 2). In fact, autoantibodies against citrullinated histone H4, H2A and H2B are commonly found in RA patients[116, 117]. Moreover, incomplete clearance of NETs, coupled with chronic inflammation, has already been correlated with initiation and/or development of autoimmune responses toward DNA and citrullinated proteins[114, 118]. Additionally, aside from an incomplete clearance of NETs, another reported factor leading to the production of autoantibodies against intracellular antigens is enhanced NETosis[115]. Examples of the effects of these two aberrant NETosis events may be

encountered in SLE and RA. In SLE, incomplete clearance of NETs has been observed, potentially due to the presence of DNAse I inhibitors or anti-NET antibodies that prevent DNAse I to break down NETs[114]. In contrast, enhanced NETosis was observed in the synovial fluid of RA patients[115]. Both events are likely to result in a constant stimulation of the immune system leading to autoantibody production[107].

While these findings suggest that neutrophils are essential players in the pathogenesis of periodontitis and RA, several studies investigated the relation of these cells to the oral microbiome[119]. This was especially due to the fact that specific members of this microbiota, such as *A. actinomycetemcomitans* and *P. gingivalis*, are able to mediate immune mechanisms relevant for RA[60]. One of such mechanisms is the aforementioned hypercitrullination[120]. This relates to the fact that human PADs require calcium cations to perform their citrullinating function[24]. Consequently, when human PADs are released into the calcium-rich extracellular space after a NETosis event, they will tend to exert an increased and aberrant citrullinating function[24, 120]. A particular mechanism for the release of PADs in the Ca^{2+}-rich extracellular milieu is observed in a study of *A. actinomycetemcomitans*, in which the bacterial toxin LtxA was shown to cause membrane lysis, potentially leading to the hypercitrullination scenario observed during NETosis[60] (Fig. 2). *P. gingivalis* lipopolysaccharide, on the other hand, was shown to inhibit apoptosis of neutrophils[121] and increase epithelial secretion of IL-8[104], an act that stimulates neutrophil migration towards the periodontal tissue and into the gingival crevice (Fig. 2). These events are of particular interest, since lifespan prolongation and increased migratory behavior of neutrophils can lead to an augmented and persistent immune response, which is the ideal condition for the onset of an autoimmune reaction. An additional piece of evidence for the relation between oral microbiome, neutrophils, and RA is given by several observations showing that neutrophils, in periodontal pockets, employ mainly NETosis as a defense mechanism against periodontal pathogens[110, 122, 123]. Clearly, since the molecular background of NET formation is still largely unknown, many possible hypotheses on the roles of this process remain to be evaluated, especially *in vivo* where the interplay between multi-species biofilms and the host can lead to the biological outcomes observed in autoinflammatory diseases.

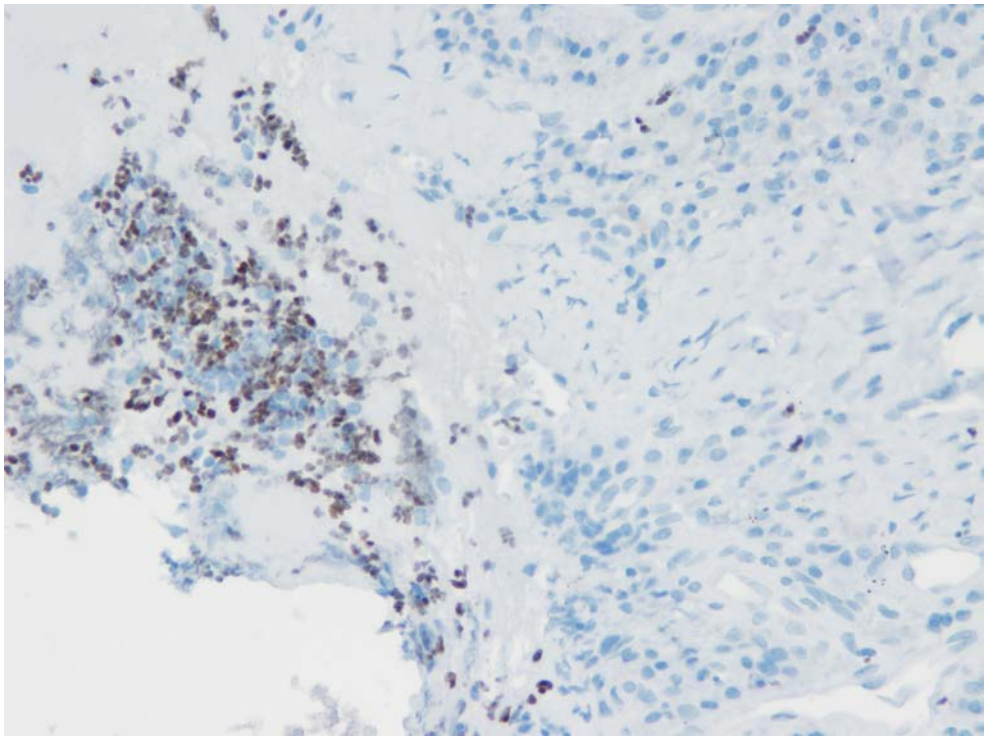

Figure 4. *Neutrophil recruitment in periodontitis.* PAD4 staining shows the enhanced recruitment of neutrophils in the periodontal tissue of a periodontitis patient. Presence of PAD4 (in brown) is indicative of neutrophil localization.

Microbial translocation to the joints

The oral cavity is not the only location where oral bacteria, and especially *P. gingivalis,* have been thought to exert a pathogenic activity. Translocation of oral bacteria to other body compartments has been evidenced as well[124-127]. While the mechanisms used by these bacteria, or their components, to reach distant locations in the body have not been completely elucidated, several hypotheses have been postulated. For instance, a direct entry of oral bacteria into the bloodstream has been proven during common dental practices, such as teeth brushing, flushing, and mastication[128, 129]. This entry mechanism seems to be enhanced under inflammatory conditions, such as periodontitis, due to higher proliferation and dilation of the periodontal vasculature. A recent study showed that oral bacteria could be found in the liver and spleen of mice with experimentally induced periodontitis. Notably, this bacterial translocation stopped after tooth removal and healing of gingival tissues, suggesting that periodontal bacteria can disseminate during breakdown of the oral barrier[89]. Another proposed mechanism of bacterial translocation is the use of host cells as a 'Trojan horse'[130]. *P. gingivalis*, in fact, is known to survive intracellularly within several cell types, such

as macrophages and dendritic cells, both of which can subsequently enter the blood stream and have the potential to disseminate bacteria throughout the body[131, 132]. Microbial translocation is of interest in the context of RA, due to the fact that immune activation mechanisms and local inflammation could occur in response to the presence of oral bacteria or their components in the synovial joints[133, 134] (Fig. 1). Corroborating this hypothesis, several studies have demonstrated the presence of *P. gingivalis, P. intermedia* and *F. nucleatum* DNA in the synovial fluid of RA patients with periodontitis[135-137]. *P. gingivalis* DNA has also been found in the joints of a murine collagen-induced arthritis model infected with "red complex" bacteria. Perhaps more remarkably, the presence of DNA of oral bacteria in the synovial joints was found to associate with arthritis exacerbation[134]. Although translocation of viable oral bacterial cells to the joint compartment of RA patients appears to be plausible, as shown for atherosclerotic plaques[138], transport of bacterial components is a more supported hypothesis to explain the presence of genetic material of *P. gingivalis* and other oral bacteria in the joints of RA patients[135].

The influence of the oral microbiome on the gut in the context of RA

Albeit infrequently, oral bacteria have been implicated in non-oral infections, among them intra-abdominal and intra-cranial sites, the appendix, and the lungs[139]. In particular, mobilization of bacteria from the oral compartment to distant physiological sites may involve the gastrointestinal tract, as humans continuously swallow oral bacteria[140-142]. This view is supported by the incidental detection of oral bacterial DNA in human feces samples (Harmsen HMJ, unpublished observations). In recent years, the impact of the oral microbiome on the gut microbial composition has been investigated in several disease scenarios. Intriguingly, oral bacteria, including *P. gingivalis*, *A. actinomycetemcomitans,* and *Fusobacterium* spp. have been implicated in several gastrointestinal diseases including pancreatic and colorectal cancer (CRC)[143]. Moreover, Atarashi *et al.*, showed in germ-free mice that oral bacteria are capable of colonizing the gut, causing chronic inflammatory reactions in predisposed hosts[144]. In the case of *P. gingivalis*, however, Geva-Zatorsky *et al*. recently suggested that this bacterium is not capable of colonizing the gut of germ-free mice since it could not be cultured from the feces of these mice[145]. Nonetheless, there is a possibility that this bacterium could remain viable during its passage through the acidic environment of the stomach due to its strong

resistance to acid[146] and therefore, under proper conditions, potentially establishes a foothold in the human intestine. Interestingly, oral administration of *P. gingivalis* had significant repercussions on the bacterial composition of the gut, specifically decreasing the proportion of Bacteroidetes and increasing the proportion of Firmicutes[146]. This, together with the fact that *P. gingivalis* was found to lower the complexity of gut bacterial communities, suggests a role for this bacterium in modulating the gut microbiome[147]. Similarly, oral administration of *A. actinomycetemcomitans* was shown to modulate the gut microbiome of mice, a process that was correlated with metabolic and immunological changes involved in non-alcoholic fatty liver disease[148]. The implications of the above-mentioned observations are highly relevant, considering that shifts in the gut microbial composition have been shown to have a significant impact on autoimmune disorders such as RA[149]. In a murine collagen-induced arthritis model, in fact, *P. gingivalis* administration significantly changed the gut microbiome, while it simultaneously increased Th17 responses and aggravated arthritis[146]. An association between the oral and gut microbiomes was, instead, recently described for RA patients in one of the aforementioned studies[45]. These individuals presented simultaneously dysbiotic oral and gut microbiomes, with decreased oral levels of *Haemophilus* spp. and increased levels of *Lactobacillus salivarius* in the gut, both of which were partially resolved after RA treatment[45]. Taken together, these findings suggest that bacteria belonging to the oral microbiome are capable of disrupting the eubiotic state of the gut microbiome, an act that can lead to chronic inflammation and trigger or enhance RA (Fig. 1).

Gut microbiome and RA

Mucosal sites are constantly exposed to microbial challenge and are considered of great importance in the initiation and modulation of microbiome-induced inflammatory responses. Among these sites, the gut is the one that has attracted most attention in the modulation of the host metabolism and immunity, due to its massive colonization by microorganisms[2]. Dysbiosis of the gut microbiome has been shown to be related to RA pathogenesis by several studies[44]. Recently, Dorozynska *et al.* demonstrated that a partial depletion of the natural gut microbiota due to antibiotics aggravated arthritis symptoms in an RA murine model[150]. As for the previously observed correlation between oral microbiome and RA, the gut microbiome is

also linked to RA through T cell mediated immunity. In healthy individuals, a $CD4^+$ T cell subtype known as regulatory T cells (Tregs) is in balance with Th17 cells, and has an anti-inflammatory role that prevents the onset of autoimmune responses[82] (Fig. 5A). Interestingly, a recent study detected decreased levels of Tregs and elevated levels of Th17 cells in the peripheral blood of RA patients, hinting at a Th17/Treg imbalance in these patients[151]. Notably, this balance can be altered by the gut microbiome, considering that production/reduction of either Th17 cells or Tregs can be orchestrated by the gut microbiota *via* Toll-like receptor 2 (TLR2)[152]. Indeed, Tregs expressing the transcription factor Foxp3 (also called $Foxp3^+$ Tregs) are known to promote homeostasis and were found to have an increase in population size dictated by TLR2 sensing of the polysaccharide A (PSA) of *Bacteroides fragilis*, a human symbiont[153] (Fig. 5B). Moreover, Tregs expressing the hormone receptor "retinoic acid receptor-related orphan receptor γt" (RORγt) were found to play an important role in regulating inflammatory responses in the intestine[154-156]. On the other hand, gut bacteria capable of causing inflammatory effects can also be present. This is exemplified by segmented filamentous bacteria (SFB), commensal murine gut microbes that have been strongly correlated with an upregulated Th17 response in the small intestine[157] (Fig. 5). In humans, an analogous process appears to be mediated by *Bifidobacterium adolescentis*, as this bacterium was shown to be capable of inducing, alone, Th17 cell production in the small intestine of mice[158] (Fig. 5B). Another important example comes from the bacterial genus *Prevotella*. A relative increase of *Prevotella* species in the gut microbiota has been correlated with the reduction of the *Bacteroides* populations[159]. Accordingly, *Prevotella* species potentially suppress the anti-inflammatory effect of *Bacteroides* species such as *B. fragilis*. *Prevotella copri*, in fact, has been linked to an inflammatory response *via* Th17 cells in the context of RA[160]. Firstly, its prevalence in the gut microbiome of new-onset untreated RA patients was reported to be significantly more abundant, when compared to healthy individuals[159]. Secondly, *P. copri* has been proven to produce *Pc*-p27, a protein that induces reactivity of T helper 1 (Th1) cells, another RA-correlated proinflammatory T cell subset[15], in 42% of new-onset RA patients[161] (Fig. 5B). Lastly, antibodies against *P. copri* were found to be extremely specific for rheumatoid arthritis, suggesting a role for this gut bacterium in the pathogenesis of RA[161]. On the other hand, another member of the same genus, *Prevotella histicola*, has been found to suppress the inflammation in a collagen-induced murine arthritis

model[162], suggesting that different *Prevotella* species may have different effects on the pathogenesis of RA.

Remarkably, gut bacteria are also capable of forming biofilms, another phenomenon capable of altering the Th17/Tregs equilibrium. Specific biofilm components, such as amyloid fibrils[163] and DNA, which serve as building blocks in the biofilm formation, have been implicated in autoimmunity via TLR recognition and subsequent Th17 activation[164, 165]. Indeed, the study of Gallo *et al.* demonstrated that a specific type of amyloid fibrils, the curli, when irreversibly associated with bacterial DNA, triggered the production of autoantibodies in a murine lupus model, suggesting a role for chronic biofilm-producing enteric infections in the pathogenesis of SLE and other autoimmune diseases[166]. Beside directly inducing autoantibody production, curli produced by enteric bacteria also activate the so-called NLRP3 inflammasome in murine macrophages, leading to production of inflammatory interleukin IL-1β[167]. This cytokine has been implicated in the differentiation of Th17 cells[82, 151] (Fig. 5B). Additionally, another study showed that upon entry of the enteric pathogen *Salmonella enterica* in the intestines of a colitis mouse model, the produced curli activated TLR2 responses, contributing to Th1 and Th17 activation, thereby promoting gut inflammation[168].

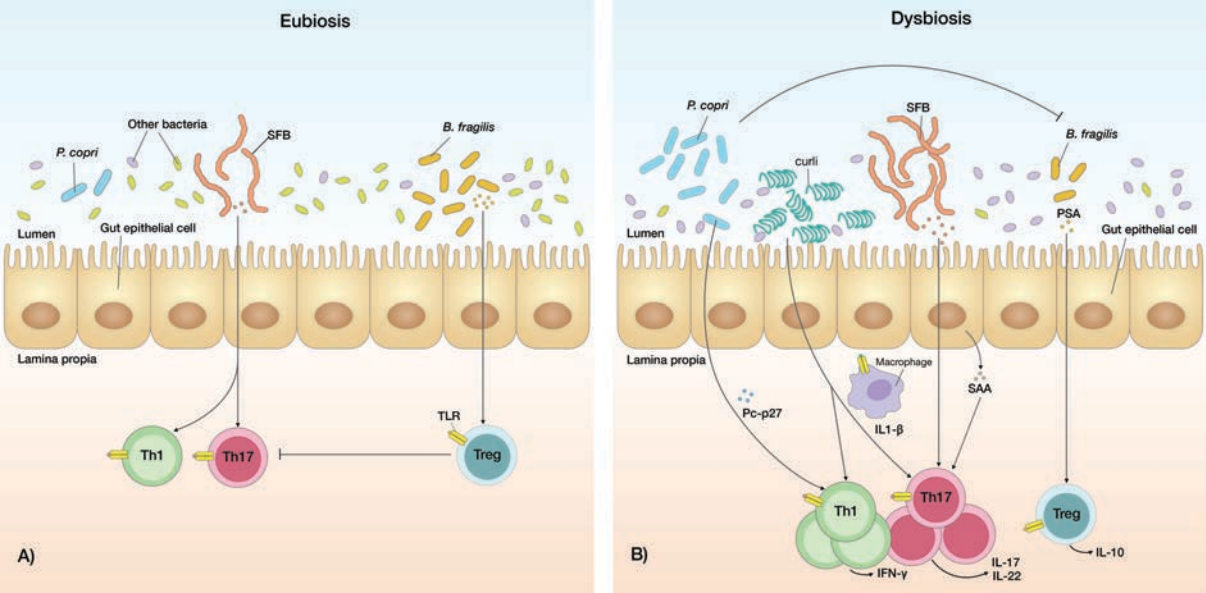

Figure 5. *Alterations of the gut microbiome composition lead to an immune imbalance.* (A) In healthy individuals, the gut microbiome is in a eubiotic state. Antigen presenting cells, including T cells, sense microbial components *via* Toll-like receptor (TLR) recognition, triggering production of balanced levels of proinflammatory and anti-inflammatory mediators. (B) Environmental, microbial and genetic factors influence the composition of the gut microbiome, leading to dysbiosis. Overgrowth of certain bacterial species, such as *Prevotella copri*, stimulates Th1 cell responses, and simultaneously inhibits the growth of *Bacteroides fragilis*, which is responsible for Treg stimulation through secretion of polysaccharide A (PSA). Moreover, bacterial curli in biofilms are sensed by TLR in macrophages, which respond by secreting IL1-β, a Th17-activating cytokine. In mice, segmented filamentous bacteria (SFB) also induce Th17 differentiation by stimulating gut epithelial cells to produce serum amyloid A (SAA). Proinflammatory cytokines and T helper cells can disseminate throughout the body *via* the blood stream and can, therefore, promote inflammation and autoimmunity in genetically predisposed individuals.

Nevertheless, a recent study using a mouse model of colitis also revealed that epithelial barrier integrity, which is essential for gut homeostasis, is promoted by recognition of enteric bacterial curli by TLR2[169]. Therefore, whether bacterial curli exert a protective or pathogenic role must depend on other factors, such as the phagocytic capacity of macrophages to digest and clear these biofilm components[169]. In this respect, our understanding of bacterial amyloids and their roles in the gut microbiome and the pathogenesis of autoimmune diseases needs to be expanded. Remarkably, however, bacteria are not exclusive when it comes to amyloid production. Human amyloids have been extensively studied in relation to several neurodegenerative diseases, such as Alzheimer's and Parkinson's disease[170]. Similarly to the study of Gallo *et al.*, other studies showed that nucleic acid-containing amyloid fibrils from human origin were also implicated in SLE pathogenesis[171]. In this context, it is particularly noteworthy that the microbiome was recently purported to play a potential role in the production of amyloids by human epithelial cells, specifically the acute phase protein serum amyloid A (SAA), which was shown to modulate neutrophil migratory behaviors and to induce

Th17 responses in the gut[172, 173]. Altogether, pathogenic and commensal members of the gut microbiome have been shown to produce amyloid fibrils in biofilms, while at the same time they are capable of stimulating SAA production by human intestinal cells, promoting a proinflammatory state (Fig. 5B).

Microbiome-based therapeutic management of RA

Despite the exponentially growing number of discoveries in the field of microbiomes, limited applications for new therapeutic avenues in the treatment of RA have been reported. However, pharmaceutical companies are expressing an increased interest in how to manipulate the microbiome to achieve positive health changes. With the understanding of the exact mechanisms by which specific microbes interact with one another and with the human host, future therapeutic strategies could aim at delivering specific bacteria to restore eubiosis, or ameliorate the effects of a dysbiotic microbiome. The microorganisms capable of conferring beneficial aspects to the host, when administered in adequate amounts, are termed probiotics[46, 174]. An interesting example of this is the aforementioned case of *P. histicola*. This probiotic might prove itself a potential therapy for RA, as it was shown to suppress arthritis in humanized mice *via* mucosal regulation, more specifically the generation of Treg cells[162]. Recently, the probiotics *Lactobacillus paracasei* and *Lactobacillus casei* have garnered special attention since they were shown to decrease inflammatory events by selectively degrading proinflammatory cytokines through their protease, lactoceptin[175]. In the case of RA, it was reported that oral administration of *L. casei* resulted in decreased Th1 effector functions in a murine collagen-induced arthritis model[176]. Similar observations were made in humans, where *L. casei* was given to RA patients who subsequently showed a significant decrease in proinflammatory cytokines, resulting in a lower disease score compared to untreated patients[177]. Another meaningful contribution to this field is the study of Vong *et al.*, in which it was demonstrated that the probiotic bacterium *Lactobacillus rhamnosus* inhibited formation of pathogen-induced NETs[178]. Vong *et al.* also demonstrated the differential capacity of different gut microbiome subsets to elicit neutrophil activation and NETosis[179]. Even though beneficial characteristics have been associated with the use of probiotic bacteria, a full-fledged status as adjunctive therapy remains to be fully

substantiated[180, 181]. Only few studies, in fact, investigated the possibility of translating the beneficial effects that probiotics have on RA animal models to humans[182, 183].

Considering the importance of a healthy gut microbiome in relation to autoimmune diseases, fecal microbial transplantation (FMT) has also been considered as a potential therapy for RA. However, while FMT proved highly effective for certain diseases, such as infectious colitis[184], it still presents several challenges, especially in not yet established therapeutic applications. The positive results obtained by studies investigating the effects of FMT in autoimmune diseases such as irritable bowel disease, however, suggest a potential future for this technique in the therapeutic landscape of RA[44, 185, 186]. Taken together, these results indicate that mechanistic studies on diverse commensal bacteria will very likely lead us to the discovery of new species involved in tissue homeostasis and these, in turn, might possibly be used as a prevention treatment for autoimmune diseases such as RA. Nonetheless, since the gut microbiota is greatly influenced by multiple factors, the identification of microbiome-based approaches that can be used universally to treat or prevent a disease still remains a challenge. In particular, genomic makeup and diet can profoundly influence the microbiome composition, making them potential obstacles when devising a universal probiotic therapy. This sparks the need for more personalized approaches[187, 188]. On the other hand, the possibility that a given microorganism might not be capable of surviving its passage throughout the stomach acids, or of effectively colonizing a target niche that is already occupied by an endogenous microbial population, poses a potentially greater challenge[189, 190]. To overcome these hurdles, particular chassis bacteria may have to be chosen after extensive research on the microbial ecology of the target niche, or be re-engineered to better suit the therapeutic needs[189]. For example, recent studies in mice have shown that bacterial strains engineered to metabolize a peculiar dietary component are capable of engrafting themselves into the already established gut microbial community when administered in concomitance with the respective dietary component[191, 192]. Another factor that should be accounted for when devising a microbiome-based therapy is the variation in host responses elicited by different strains of the same species. This is underscored by studies of Geva-Zatorsky *et al.*, who demonstrated that certain *Bacteroides* species display strain-specific differential immunomodulatory capacities[145]. Considering these challenges, the use of small molecules mimicking the interaction between beneficial bacteria and the host could represent a better alternative for therapy[193]. In this respect, an identified bacterial molecule of interest is the

aforementioned PSA from *B. fragilis*, which was shown to induce the maturation of the host immune system and to elicit protective effects against colitis by promoting the production of Foxp3$^+$ Treg cells[153, 194]. Based on these findings, a PSA-based oral therapy was created to treat autoimmune, inflammatory, and allergic diseases[193].

An alternative potential microbiome-based therapy for RA targets the pathogens implicated in the etiology of this disease, such as *P. gingivalis*. For example, a recent study in mice with experimental arthritis showed amelioration of the symptoms of both periodontitis and collagen-induced arthritis upon treatment with a FimA antibody[195]. This antibody, targeting the major fimbrillin protein of *P. gingivalis*, appeared to attenuate bacterial attachment and aggregation on the tested murine fibroblasts[195]. An additional potential avenue for therapy concerns the formation of biofilm by oral bacteria. New compounds capable of inhibiting quorum sensing molecules, such as AI-2, have been recently tested both *in vivo* and *in vitro*. Perhaps due to the novelty of this field, few studies have investigated the beneficial effects of quorum sensing inhibitors[196, 197]. However, the potential therapeutic effect they demonstrated in periodontitis, which is mainly due to their capability to limit biofilm formation, shows some promise for eventual future applications in the treatment of RA. Nevertheless, with the important and very rapid advances in the "omics" field and the development of massive computational approaches, the translational potential of emerging therapeutic agents has become increasingly more evident. To support this, in the case of RA, Tieri *et al.* developed a multi-omic map to estimate the outcomes of novel therapies focusing on several aspects of RA, including immune responses mediated by the gut microbiome, leading to potential targets for RA treatment[198]. Overall, as the scientific community continues to elucidate the complex relationships between the microbiome and human health, numerous therapeutic targets are being identified which, in turn, may be efficiently tested *in silico* to predict, to some extents, the outcome of future clinical trials. Hopefully, advances as summarized above will bring us one step closer to the discovery of a 'universal' therapy to prevent or treat RA and other autoimmune diseases.

Concluding Remarks

Over the past decade, the field of microbiome research was exponentially expanded by more and more studies demonstrating the profound impact of the microbiome on human health

and disease. Moreover, important technological approaches have advanced our understanding of how the microbiome and the host interact to maintain tissue homeostasis. Yet, major knowledge gaps still exist. For instance, the functional consequences of microbial biofilm formation need to be evaluated in more detail, because recent studies have provided evidence that biofilm-related phenomena and components influence the immune system. A second important area that needs to be explored more in depth concerns the microbial modulation of neutrophil activity, especially since neutrophils could represent a key source of autoantigens in RA patients. In particular, recent observations imply that the oral microbiome influences NETosis. However, further insight is needed to pinpoint the microbial populations responsible for altering neutrophil activities, potentially leading to the development of microbial therapies that restore neutrophil homeostasis. Luckily, novel 'omics'-based technologies to study the microbiome and its interactions with the host have recently been developed. These can now be exploited to overcome the limitations of classical biochemical and immunological approaches. Moreover, innovative computational tools will allow us to predict and verify the outcomes of potential therapies. As a consequence, we will surely experience, in the near future, a boost of preventive and therapeutic approaches to promote, balance, or restore the beneficial interactions between the microbiome and the human body.

Ethics approval

Figure 4 was recorded in the context of a previous study that received Institutional Review Board approval from the Medical Ethics Committee of the University Medical Center Groningen (METc UMCG 2007.195). This study was performed in accordance with the guidelines of the Declaration of Helsinki and the institutional regulations, and all samples were anonymized.

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