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The microbiome in autoimmune rheumatic disease

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Abstract

Microbial contributions to the immunopathogenesis of autoimmune rheumatic diseases have been studied since the advent of germ theory in the 19th century. With the exception of Group A *Streptococcus* in rheumatic fever, early studies failed to establish causal relationships between specific pathobionts and rheumatic disease. Today, systemic autoimmune diseases are thought to result from a complex interplay of environmental factors, individual genetic risk, and stochastic events. Interactions of microbiota and the immune system have been shown to promote and sustain chronic inflammation and autoimmunity. In mechanistic studies, microbe-immune cell interactions have been implicated in the initiation of autoimmune rheumatic diseases, e.g. through the posttranslational modification of autoantigens in rheumatoid arthritis or through neutrophil cell death and cross-reactivity with commensal orthologs in systemic lupus erythematosus. In parallel, modern molecular techniques have catalyzed the study of the microbiome in systemic autoimmune diseases. Here, I review current insights gained into the skin, oral, gut, lung, and vascular microbiome in connective tissue diseases and vasculitis. Mechanism relevant to the development and propagation of autoimmunity will be discussed whenever explored. While studies in connective tissue diseases and vasculitis have almost invariably shown abnormal microbiome structure (dysbiosis), substantial variability in microbial composition between studies makes generalization difficult. Moreover, an etiopathogenic role of specific pathobionts cannot be inferred by association alone. Integrating descriptive studies of microbial communities with hypothesis-driven research informed by immunopathogenesis will be important in elucidating targetable mechanisms in preclinical and established rheumatic disease.

Keywords

microbiome; pathobionts; connective tissue disease; rheumatoid arthritis; Sjögren's syndrome; systemic sclerosis; scleroderma; vasculitis

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Conflict of Interest

None

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Introduction

The systemic autoimmune diseases are a heterogeneous group of disorders characterized by self-sustained, autoreactive adaptive immune responses resulting in immune-mediated end-organ damage. The etiology of these diseases is almost invariably unknown, but a complex interplay of variable genetic risk, environmental factors, and stochastic events is thought to result in loss of immunological tolerance and autoimmunity[1].

In an individual patient, autoimmunity doesn't develop in isolation, but in the context of the entirety of the human microbiota, a symbiosis of an estimated 10-100 trillion microbial cells that includes bacteria, yeast, archaea, protozoa, and infecting viruses[2,3]. Given co-evolution over millennia, it is not surprising that the microbiome has a primary role in human health and disease[4]. Compositional and/or functional alterations of the microbiome, often designated dysbiosis, are associated with autoimmune and inflammatory diseases[5,6], and experimental evidence suggest that the microbiome can shape adaptive immune responses and autoimmunity[7]. The balance of T helper cell subsets and regulatory T cells has been shown to be affected by specific microbial species. Colonization with segmental filamentous bacteria (SFB) in mice is sufficient to induce Th17 cells, thereby propagating inflammation[8]. *Clostridia* in contrast can induce regulatory T cells[9,10], thus dampening autoimmune responses[11].

The concept that pathobionts may initiate autoimmunity in systemic autoimmune diseases is not novel. Even preceding concepts of autoimmunity, bacterial species were proposed to be causative agents in many rheumatic diseases including rheumatic fever and rheumatoid arthritis (RA)[12-16]. While these early studies successfully identified "*Micrococcus rheumaticus*" (now *Streptococcus pyogenes*) as the etiologic agent that initiates rheumatic fever ("acute rheumatism") in the susceptible host[12], the discovery of a pathogen relevant to "chronic rheumatism" was not successful. Importantly, prolonged preclinical autoimmunity (as is the case for RA) makes the identification of relevant microbial species based on temporal association to exposure almost impossible[17]. Similarly, a one pathogen – one disease model is unlikely to apply to host-microbiota interactions propagating systemic autoimmune diseases.

Today, significant advances in our understanding of rheumatic disease immunopathogenesis have provided a mechanistic framework to study host-microbiota interactions and query candidate pathobionts. Indeed, the study of candidate pathogens in RA has been shaped by our understanding of risk (periodontitis) and their putative roles in autoantigen citrullination[13,18]. Similarly, understanding of early antigenic determinants during preclinical disease has informed studies on bacterial orthologs as drivers of the anti-Ro60 autoantibody response in lupus[19]. Moreover, advances in sequencing technologies have allowed for unprecedented studies of microbiota composition in a large number of patients. Insights gained from these emerging studies will be reviewed here for the connective tissue diseases and primary vasculitides with the goal of facilitating comparison across the spectrum of autoimmune rheumatic disease.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by synovial inflammation, joint erosion, and the presence of anti-citrullinated protein antibodies (ACPA) [20]. A bacterial role in the immunopathogenesis of RA has been hypothesized since the advent of germ theory but attempts to identify a single relevant species did not yield consistent results[13-16]. The discovery of genetic susceptibility alleles (HLA-DRB1 shared epitope), environmental risk factors (smoking, periodontitis, among others), and posttranslational protein citrullination as a primary determinant of autoantibody reactivity in RA has since provided a mechanistic framework to understand microbial contributions to RA pathogenesis[21]. Several mucosal surfaces have been implicated as sites of disease initiation in RA, including the periodontium, the lungs, the gut, and the genitourinary system[22]. Over the past decade, both hypothesis-driven research and “unbiased” sequencing-based approaches have generated unique mechanistic insights into the role of specific pathobionts in RA. Through these studies, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Prevotella copri* have emerged as lead candidate pathogens in RA[13,23,24].

Mechanistic studies

Periodontitis is a chronic inflammatory disease of the gums whose etiopathogenesis has been linked to local bacterial dysbiosis involving several so-called periodontal pathogens[25]. An association of RA and periodontitis was already observed in the early 20th century, when the existence of a periodontal pathogen with relevance for both periodontal infection and RA was postulated[16]. This increased prevalence of periodontitis in RA was confirmed in the modern era, although the relative risk increase observed in meta-analyses is relatively small[26].

Porphyromonas gingivalis

P. gingivalis is a keystone pathogen in periodontitis and the most extensively studied periodontal pathogen in RA. A role for *P. gingivalis* in RA was first proposed because of its expression of a bacterial protein arginine deiminase (PPAD) that can citrullinate free L-arginine and C-terminal arginine residues in cleaved peptides[18]. This is distinct from calcium-dependent human protein arginine deiminases (PADs) that modify internal arginine residues within uncleaved proteins[27]. C-terminal citrullination of bacterial and host protein has been hypothesized to break immunological tolerance and initiate the ACPA response in RA[28]. It remains unknown whether a single C-terminal citrullination site, as generated by PPAD, is sufficient to induce ACPAs[29]. Many studies have investigated the clinical association of *P. gingivalis* and RA, most commonly by measuring serum antibody reactivity, by molecular detection of bacterial DNA from plaque or gingival crevicular fluid, or by bacterial culture[29]. A role of *P. gingivalis* in the development of experimental arthritis has been explored in both rat and mouse models, and exacerbation of experimental arthritis has been reported in several of these studies[23]. Given the inability of most mouse and rat strains to make antibodies against citrullinated proteins[30,31], possibly due to lack of relevant MHC class II susceptibility alleles, the mechanism underlying arthritis

exacerbation in these models is not entirely elucidated, but may involve complement subversion, Toll-like receptor activation, and skewing toward proinflammatory Th17 immune responses, among others[25,29,32]. The mechanistic studies linking *P gingivalis* and RA have recently been reviewed in detail[23,29].

Aggregatibacter actinomycetemcomitans

A role for *A actinomycetemcomitans* in RA pathogenesis was first proposed in 2016 in studying hypercitrullination of gingival crevicular fluid in patients with periodontitis[13]. Citrullinated RA autoantigens identified in gingival crevicular fluid invariably showed modification of internal arginine residues, excluding citrullination by a bacterial protein arginine deiminase such as PPAD. The periodontal pathogen *A actinomycetemcomitans* was shown to induce cellular hypercitrullination by activating endogenous PADs in human neutrophils. This process is mediated through the bacterial pore-forming toxin leukotoxin A (LtxA), which is the primary virulence factor of *A actinomycetemcomitans*[33]. LtxA binds to beta-2 integrin on the neutrophil surface, resulting in transient pore formation, calcium influx, activation of calcium-dependent intracellular PADs, and dysregulated citrullination of the neutrophil proteome[13]. LtxA induces citrullination of a wide range of RA autoantigens which are subsequently released by the dying neutrophil in a process that is reminiscent of NETosis but is biologically distinct. This unique form of cell death was coined leukotoxic hypercitrullination (LTH)[34]. Exposure to *A actinomycetemcomitans* by anti-LtxA antibody reactivity was observed in a large subset of RA[13], a finding that has been replicated in an independent Dutch cohort[35,36]. HLA-DRB1 shared epitope risk alleles associated with ACPAs only in RA patients with evidence of Aa exposure, suggesting that LtxA-induced protein citrullination may play a role in ACPA production in genetically susceptible individuals[13]. More recently, a possible role for *A actinomycetemcomitans* in ACPA production was highlighted in a patient with *A actinomycetemcomitans* endocarditis, in whom arthritis and ACPAs resolved with antibiotic treatment[37].

Prevotella copri

Contribution of the gut microbiome to RA pathogenesis has been suggested by animal models of autoimmune arthritis. Experimental arthritis in the K/BxN mouse model is highly attenuated under germ-free conditions due to decreased T helper 17 (Th17) cells, and colonization with segmented filamentous bacteria (SFB), a murine commensal, is sufficient to reestablish the autoimmune phenotype in this model via T follicular helper cell differentiation[8]. The gut microbiome in patients with RA has been interrogated by several studies. In a study of untreated new-onset RA, defined as disease duration of at least 6 weeks and up to 6 months since diagnosis, Scher et al. [24] showed enrichment of the gut microbiome for *P copri* which demonstrated a pro-inflammatory function in a mouse model of chemical-induced colitis. Intriguingly, *P copri* was not enriched in patients with established RA (disease duration >6 months). A higher abundance of *P copri* was observed in new-onset RA without HLA-DRB1 shared epitope alleles, although 100% of new-onset RA patients in this study were ACPA positive. While not replicated in a Chinese study[38], increased abundance of *P copri* among gut microbiota was observed in a recent study of 83 individuals during pre-clinical stages of RA as compared to first degree relatives of RA patients[39]. In an experimental arthritis model, inoculation of *P copri*-dominated fecal

samples from patients with RA into germ-free arthritis-prone SKG mice was associated with increased intestinal Th17 cells and worse arthritis[40]. Pianta et al. [41] showed that the HLA-DR presented peptide from *P copri*, Pc-p27, stimulated Th1 responses in patients with new-onset RA. Moreover, IgA and IgG antibody responses to Pc-p27 or whole *P copri* were observed in new-onset RA and established RA, but uncommon in other rheumatic diseases. In a second study, Pianta et al. [33] were able to show that HLA-DR-presented N-acetylglucosamine-6-sulfatase (GNS) and filamin A peptides, which were identified as RA autoantigens, showed significant sequence homology with epitopes of *Prevotella* species. RA patients with T cell reactivity to GNS and filamin A self-peptides also showed reactivity to the corresponding microbial peptides, suggesting a mechanistic link between mucosal immunity to *Prevotella* and autoimmune responses relevant to synovial inflammation in RA.

Compositional studies of the RA microbiome

The oral microbiome in RA

The composition of the oral microbiome in RA has been explored in several studies. Scher et al. [43] reported that the subgingival microbiota in patients with new-onset RA was distinct from healthy controls. The abundance of *P gingivalis* in this study was directly associated with the periodontitis severity. *P gingivalis*, however, was not correlated with ACPAs, and overall exposure to *P gingivalis* was similar between patients with new-onset RA and controls. In the same study, *Anaeroglobus geminatus* correlated with ACPAs and rheumatoid factor, and only *Prevotella* and *Leptotrichia* species were characteristic of new-onset RA irrespective of PD status and absent in controls. In a large Chinese study of patients with treatment-naïve RA and healthy controls, there was concordance of the gut and oral microbiome[38]. Dysbiosis was observed in both gut and oral microbiomes in RA, and this partially normalized with DMARD treatment (most commonly methotrexate). *Haemophilus* spp. were decreased in RA and negatively correlated with serum autoantibodies, while *Lactobacillus salivarius* was overrepresented in RA and in patients with high disease activity. Neither *P gingivalis* nor *Aggregatibacter* spp. were found to be enriched in RA.

Examining the subgingival microbiome of 78 patients with established RA, periodontitis was diagnosed by comprehensive periodontal examination in 82% of RA (mild 9%, moderate 55%, severe 18%)[44]. The subgingival microbiome in patients with active disease differed from RA in remission. Despite high prevalence of periodontitis, *P gingivalis* in subgingival plaque was detected in only 14% of patients, and was associated with increased pocket depth and CRP, but not ACPAs. Current treatment with oral glucocorticoids, current smoking, and periodontal status were all associated with alterations of microbiota composition.

In a study of the salivary microbiome in 110 RA patients and 67 osteoarthritis patients, taxa that were more abundant in RA as compared to osteoarthritis included *Neisseria subflava*, *Haemophilus parainfluenzae*, *Veillonella dispar*, *Prevotella tannerae*, *Actinobacillus paraaemolyticus*, *Neisseria*, *Haemophilus*, *Prevotella*, *Veillonella*, *Fusobacterium*, *Actinobacillus*, and *Aggregatibacter*[45].

A large study by Mikuls et al. [46] examined the subgingival microbiome in 260 patients with RA and 296 patients with osteoarthritis by 16S ribosomal RNA (rRNA) gene sequencing. Notably, subgingival microbiota did not reliably discriminate RA from osteoarthritis patients in this study. Decreased abundance was observed for 10 operational taxonomic units in RA compared to osteoarthritis, including periodontal pathogens *Peptostreptococcus*, *Porphyromonas*, *Prevotella* and *Treponema* spp. A history of smoking, non-Caucasian race, and non-married status were associated with overabundance of pathobionts.

Interrogating the microbiome of periodontally healthy individuals with and without RA, the subgingival microbiota differed significantly between RA and healthy individuals[47]. In the absence of periodontitis, *P. gingivalis* and *A. actinomycetemcomitans* did not differ significantly between groups. In contrast, *Cryptobacterium curtum* was enriched in RA in this study. While the authors suggest a role for *C. curtum* in RA pathogenesis due to its ability to convert arginine to L-citrulline, ACPAs recognize peptidylcitrulline in the context of specific peptide motifs and not the free amino acid L-citrulline[29].

In a recent study of 42 RA patients and 47 non-RA subjects with known periodontitis status, 86% of RA with periodontitis were ACPA positive compared to 33% of RA without periodontitis. Analysis of the subgingival microbiome demonstrated that non-periodontitis RA patients had an increased bacterial burden compared to healthy individuals without periodontitis. Similarly, RA with periodontitis was associated with increased microbial diversity when compared to non-RA periodontitis patients. RA patients without periodontitis showed enrichment in periodontitis-associated bacteria such as *Prevotella* species (*P. melaninogenica*, *P. denticola*, *P. histicola*, *P. nigrescens*, *P. oulorum*, *P. maculosa*), among other pathobionts. Interestingly, RA patients with periodontitis showed an increased abundance of pathogens including *A. actinomycetemcomitans*, *Prevotella* spp., and *Parvimonas micra* when compared to non-RA periodontitis. Salivary cytokine analysis showed increased levels of inflammatory cytokines in RA versus controls. IL-17 was markedly increased in RA with periodontitis, but not periodontitis without RA[48].

Studies of oral and subgingival microbiota composition in RA are characterized by considerable heterogeneity but demonstrate anticipated alterations in abundance of periodontitis-associated bacteria (*A. actinomycetemcomitans*, *P. gingivalis*, *Prevotella*, among others) with periodontal disease status and severity. Moreover, the subgingival microbiome associated with periodontitis in RA may quantitatively differ from periodontitis in individuals who do not have RA, as suggested by a low abundance of *P. gingivalis* [38,44,46] and an expansion of microbial diversity and certain periodontal pathogens (*A. actinomycetemcomitans*, *Prevotella* spp., and *P. micra*) in RA patients with periodontitis compared to periodontitis in individuals that do not have RA[48]. These studies may further suggest significant differences in microbiota structure between continents and/or ethnicities. Finally, identifying pathobionts with relevance to the immunopathogenesis of RA from these compositional studies may be difficult (if feasible at all), highlighting the relevance of mechanistic research in the study of the microbiome in RA.

The pulmonary microbiome in RA

Lung involvement is a common extraarticular manifestation of RA and can present as interstitial lung disease (ILD), lung nodules, bronchiectasis, and/or pleural effusions. Cigarette smoking is a well-established risk factor for RA[21], but mechanisms mediating this risk are still ill defined. The lung represents as a potential site of disease initiation in RA[22], and the finding of sputum IgA ACPA in RA and first-degree relatives may support this hypothesis[49]. Despite this, relatively little is known about the lung microbiome in RA. Scher et al. examined the composition of microbiota in bronchoalveolar lavage (BAL) fluid of patients with early, DMARD-naïve RA and compared these to patients with lung sarcoidosis and healthy controls[50]. The lung microbiome in RA was overall less diverse and abundant when compared to health, but showed significant similarities to sarcoidosis. The presence of the genus *Prevotella* correlated with RF IgA and ACPA fine specificities, while *P. gingivalis* was underrepresented in RA BAL when compared to health controls. While the study provides a glimpse into the lung microbiome in RA, mechanistic studies may be needed to characterize the interplay of microbiota, smoking, immune cells, and autoimmunity in the RA lung.

Sjögren's syndrome

Sjögren's syndrome (SS) is a connective tissue disease characterized by autoantibody production (most commonly directed against ribonucleoproteins TRIM21/Ro52/SS-A, Ro60, and La/SS-B), autoimmune destruction of lacrimal and salivary glands, and a wide range of extraglandular disease manifestations[51]. Reduced production of saliva and tear film, rich sources of innate antimicrobial proteins and antibodies, results in alteration of mucosal barrier function and may favor dysbiosis and colonization with pathobionts[52]. Dysbiosis and bacterial translocation have been hypothesized to propagate local and systemic inflammation and modulate disease severity in SS. Several descriptive studies have explored alterations of the oral and gut microbiome in SS in recent years. Moreover, elegant work by Greiling et al. [19] identified commensal orthologs of Ro60 as possible drivers of anti-Ro60 autoantibodies in lupus, providing needed mechanistic insights into the role of microbiota as initiators of autoimmunity in connective tissue diseases. Interestingly, *Corynebacterium amycolatum* has been shown to colonize the lacrimal duct, making *C. amycolatum* Ro60 a candidate ortholog for the development of anti-Ro60 antibodies in SS.

Van der Meulen et al. [53] examined the bacterial composition of the oral microbiome in 37 patients with primary SS (pSS), 86 sicca patients without SS, and 24 healthy controls by 16S rRNA sequencing. The buccal mucosa microbiome of pSS and non-SS sicca patients differed from healthy controls, with a higher *Firmicutes/Proteobacteria* ratio observed in both pSS and non-SS sicca patients. When adjusting for salivary secretion rate, no taxon was specifically associated with pSS as compared to non-SS sicca. These studies highlight how oral dryness may favor dysbiosis. In a similar study interrogating chewing-stimulated whole saliva, several periodontal pathogens including *Streptococcus intermedius*, *Prevotella intermedia*, *Fusobacterium nucleatum vincentii*, *Porphyromonas endodontalis*, *Tannerella* spp., and *Treponema* spp. were only detected in the context of oral dryness (pSS and non-SS sicca patients), while *Porphyromonasasteri* showed increased abundance in healthy

controls[54]. Comparing microbial composition in oral rinse samples of 22 patients with pSS and 23 healthy patients, *Veillonella* was found to be enriched in pSS, while *Actinomyces*, *Haemophilus*, *Neisseria*, *Rothia*, *Porphyromonas*, and *Peptostreptococcus* were less abundant compared to oral health[55]. Whether oral dysbiosis is merely a consequence of oral dryness and decreased antimicrobial properties of saliva or an active driver of systemic and target tissue inflammation in patients with SS is still unknown.

The intestinal microbiome in SS has been investigated by de Paiva and colleagues. Inducing intestinal dysbiosis by antibiotic therapy in a mouse model of keratoconjunctivitis sicca, they found that exposed mice had worse dry eye disease (conjunctival CD4+T cell infiltration, goblet cell loss, corneal barrier disruption) when compared to control mice. This was associated with a decreased abundance in *Clostridium* and increased *Enterobacter*, *Escherichia/Shigella*, and *Pseudomonas* abundance. Analyzing conjunctival, oral, and fecal samples from 15 SS patients and controls, they found that the severity of SS ocular and systemic disease (but not systemic disease alone) as assessed by EULAR Sjögren's syndrome disease activity index (ESSDAI) was inversely correlated with fecal microbial diversity. Overall, intestinal dysbiosis in SS was characterized by low relative abundance of commensal bacteria (*Bacteroides*, *Parabacteroides*, *Faecalibacterium*, and *Prevotella*) and high relative abundance of potentially pathogenic genera (*Pseudobutyrvibrio*, *Escherichia/Shigella*, and *Streptococcus*). No differences in ocular surface microbiota were observed[56]. Mandl et al. [57] identified intestinal dysbiosis when studying 42 consecutive patients with pSS and 35 matched healthy controls using a genome-based microbiota test (GA-map) limited to 54 bacterial ribosomal RNA probes. Severe dysbiosis as defined by this commercial test was more prevalent in pSS (21% vs 3%), and associated with higher ESSDAI total score, decreased complement C4, and increased fecal calprotectin compared to remaining pSS patients.

Together, these studies suggest that the severity of ocular and systemic features of SS may associate with intestinal dysbiosis, although the directionality of this relationship is uncertain in humans. Interactions of the oral and gut microbiome in SS remain to be defined. The alterations of physiological mucosal barrier function in SS may favor colonization with pathobionts or commensals that may elicit immune responses to ubiquitously expressed self-proteins. These studies provide a promising framework to study the origins of the autoantibody in SS and lupus.

Systemic sclerosis

Systemic sclerosis (SSc) is a multiorgan system connective tissue disease characterized by vasculopathy and progressive fibrosis, most prominently affecting the skin, the lungs, and the gut. Structural and function changes of these body surfaces may profoundly alter the established microbiome. Conversely, it is conceivable that dysbiosis may favor SSc pathogenesis and severity. This complex interrelationship has only recently been explored by applying modern sequencing techniques.

Gastrointestinal involvement is observed in a majority of patients with SSc and can present with dysmotility (esophageal dysmotility, gastroparesis, and delayed small and large

intestinal transit), vasculopathy (gastric antral vascular ectasia, angiodysplasia), and their complications, including small intestinal bacterial overgrowth, malabsorption, and pseudo-obstruction[58]. Reasons for abnormal microbiota in SSc are manifold. Alterations in peristalsis may favor dysbiosis and systemic bacterial translocation, both of which have the potential to alter systemic immune responses[6,59-61]. Vasculopathy may alter mucosal barrier function, integrity, and gut homeostasis. Indeed, intestinal-type fatty acid-binding protein (I-FABP), lipopolysaccharide (LPS), and soluble CD14, markers of enterocyte damage, microbial translocation, and immune system activation, respectively, are elevated in sera of SSc patients compared to healthy controls. Higher levels of LPS were associated with small intestinal bacterial overgrowth[62]. Finally, patients with symptomatic gastrointestinal disease may resort to or be treated with dietary changes, and intermittent antibiotic therapy to ameliorate symptoms of small intestinal bacterial overgrowth is not uncommon[63,64].

Published studies exploring the gut microbiome in SSc have invariably found dysbiosis[65-68]. Volkmann et al. [66], studying fecal samples in 34 SSc patients from two cohorts (USA, Norway), reported lower relative abundance of the commensal genera *Bacteroides* (observed in both cohorts), *Faecalibacterium* (US), *Clostridium* (Norway). *Clostridium* species have been shown to induce expansion of regulatory T cells[9]. Whether this putative immune-regulatory mechanism contributes to an observed association between increased *Clostridium* abundance and less gastrointestinal symptoms remains to be defined. *Prevotella* was more abundant in SSc patients with severe gastrointestinal symptoms[66]. *Prevotella* spp. have previously been shown to promote Th17-mediated immune responses, and *Prevotella copri* enhances chemical colitis in mice[69]. An increased relative abundance of the pathobiont *Fusobacterium* was also observed in the US cohort[66].

Patrone et al. [68] studied the composition of the intestinal microbiota in 9 SSc patients with and 9 patients without gastrointestinal involvement. SSc patients with gastrointestinal involvement demonstrated an increase in *Lactobacillus*, *Eubacterium*, and *Acinetobacter*, but decreased abundance of *Roseburia*, *Clostridium*, and *Ruminococcus* as compared to controls. Compared to SSc patient with gastrointestinal disease, gut microbiota in SSc without gastrointestinal involvement were more similar to the microbiota of healthy subjects, with the exception of an overrepresentation of *Streptococcus salivarius*.

Andréasson and colleagues [65] studied dysbiosis in 98 consecutive patients with SSc using a limited genome-based microbiota test (GA-map). In their cohort, 76% of SSc showed dysbiosis as defined by this assay. Dysbiosis was more common in patients with esophageal dysmotility and was associated with risk of malnutrition. Several clinical features including telangiectasias, pitting scars, and interstitial lung disease were also associated with dysbiosis. These studies highlight distinct shifts in the composition of gut microbiota in SSc, but small samples sizes and considerable variability between cohorts are prohibitive to make definitive conclusions. Whether dysbiosis in SSc represent a primary alteration relevant to disease etiopathogenesis or secondary perturbations of the gut microbiome due to local or systemic immune dysregulation, intestinal end-organ involvement (fibrosis, vasculopathy), dietary changes, or immunosuppressive and/or antibiotic treatment is unknown and remains speculative.

The skin microbiome in SSc has similarly attracted research interest. In a study of 4 patients with diffuse cutaneous SSc, RNA-seq was employed to quantify non-human sequencing reads in forearm skin. No differences were observed with regards to bacterial and viral read counts, but the environmental yeast *Rhodotorula glutinis* was highly enriched in SSc skin as compared to normal skin of healthy controls[70]. *R. glutinis* has a role as an emerging opportunistic human pathogen, but *Rhodotorula spp.* are ubiquitous environmental contaminants and the most common yeast covered from the hands of nurses[71]. In a recent study, Johnson et al. [72] examined lesional and non-lesional skin from 23 SSc patients by RNA-seq. Skin from SSc patients showed changes in microbial composition compared to controls, with decreases in *Propionibacterium* and *Staphylococcus*, and increases in gramnegative taxa, including *Burkholderia*, *Citrobacter*, and *Vibrio*. Differences did not associate with disease subtype, and limited and diffuse SSc skin demonstrated similar abundances of major taxa. Interestingly, lesional skin and non-lesional skin demonstrated no differences in microbial composition[72], despite collection from distant sites that show substantial variation in microbial composition in healthy subjects (forearm vs back)[73]. *Rhodotorula* was similarly detected in both SSc and controls[72], not replicating the enrichment observed prior[70]. Gene expression of epithelial antimicrobial peptide β -defensin 1 was significantly lower in SSc lesional skin as compared to controls and non-lesional SSc skin[72].

The lung microbiome and its relation to SSc-ILD has not been explored. Future studies may address whether progression of SSc-ILD is associated with the presence of specific microbial genera, analogous to what has been observed in idiopathic pulmonary fibrosis[74,75]. The clinical association of gut dysbiosis and ILD observed in one study is intriguing. In this context, the contributions of intestinal dysmotility and gastroesophageal reflux disease to the composition of the lung microbiome and ILD progression in SSc will be of interest.

Other Connective Tissue Diseases

The idiopathic inflammatory myopathies (IIM) comprise a larger group of autoimmune muscle disease with variable involvement of other organ systems. Dermatomyositis, antisynthetase syndrome, immune-mediated necrotizing myopathies, and inclusion body myositis are clinically distinct entities that show a strong correlation between myositis-specific antibodies and disease phenotype. To date, no data on the microbiome in inflammatory myopathies have been reported.

No currently published studies have explored the microbiome in undifferentiated connective tissue disease (UCTD), mixed connective tissue disease (MCTD), or overlap syndromes.

Vasculitis

Primary vasculitides encompass a spectrum of chronic diseases characterized by inflammation of small, medium-sized, and large vessel[76]. Several studies have explored contributions of microbiota to the pathogenesis of systemic vasculitis. Interestingly, this research has both focused on the microbiome colonizing mucosal surfaces, of obvious

interest in diseases with prominent involvement of upper and lower respiratory tract such as granulomatosis with polyangiitis (GPA), as well as potential microbial colonization of the vascular wall itself (“vascular microbiome”)[77-79]. Finally, the last few years have seen a resurgence of research (and lively debate) on the potential role of varicella zoster virus (VZV) in the etiology of giant cell arteritis (GCA). The latter will be reviewed here without extending this review to all viruses previously studied in rheumatic disease.

Giant Cell Arteritis

While blood vessels in health are classically considered free of microbial species, several studies have proposed the existence of a vascular microbiome[80]. In studying atherosclerotic disease, several bacterial species have been implicated and detected in atherosclerotic arterial tissue including spirochetes, *Chlamydia pneumoniae*, and the periodontal pathogens *P gingivalis* and *A actinomycetemcomitans*, among many others[81-85]. Some of these have been cultured from retrieved arteries[86,87]. More recently, sequencing of microbial nucleic acids has extended early studies. These studies have identified a plethora of organisms by their nucleic acids signature, raising the possibility that pathologically altered vascular beds may serve as unspecific “sieves” for bacterial remnants. Moreover, unbiased sequencing approaches are not immune to contamination during their surgical retrieval.

An infectious stimulus for GCA has been suspected on the basis of cyclical patterns of occurrence[88]. In a pioneer study of microbiota in GCA, sequences with homology to several α -proteobacteria were identified in micro-dissected inflamed temporal artery sections of patients with GCA as compared to adjacent uninvolved tissue[89]. In another study, 16S rRNA sequencing on frozen temporal arteries and serum LPS measurements suggested a role for a *Burkholderia pseudomallei*-like strain in GCA[90]. Subsequently, unbiased DNA sequencing on 17 formalin-fixed, paraffin-embedded temporal artery biopsy (TAB) samples identified *Propionibacterium acnes* and *Escherichia coli* as highly abundant microorganisms, but found no enrichment when comparing GCA to non-GCA controls, suggesting that environmental contamination with skin flora despite collection under aseptic conditions is common[77]. Enrichment of the previously invoked candidate species including *Burkholderia* or bacteria studied in atherosclerotic disease was not observed[77]. Recently, Hoffman et al. [78] utilized both fluorescence in situ hybridization (FISH) and 16S rRNA sequencing to define the microbiome of temporal arteries in GCA and controls. Differences in beta diversity, with high person-to-person variability, were observed in GCA as compared to control TABs, with largest differential abundances seen for *Proteobacteria*, *Bifidobacterium*, *Parasutterella*, and *Granulicatella*. No single pathogen characteristic of GCA was appreciated. Interestingly, bacterial 16S rRNA was also identified by FISH in the media of temporal arteries in both GCA and controls, suggesting these vascular beds may not be sterile. These findings are somewhat analogous to the detection of nucleic acids of various bacterial species in the synovium of patients with arthritis and cannot delineate the presence of live organisms (infection/colonization) from “trapping” of microbial remnants. To date, no organisms have been reproducibly recovered from GCA temporal arteries by bacterial culture. While there is little evidence to date that specific pathobionts or vascular

dysbiosis initiate or propagate vascular inflammation seen in GCA, this remains an area of active interest.

The neurotropic human herpes virus VZV can establish latent infection in dorsal root ganglia, and upon reactivation, causes herpes zoster. Less common complications of VZV reactivation include myelitis, retinal necrosis, and focal vasculopathies of the central nervous system[91]. A putative role of VZV in GCA has repeatedly been explored over the past decades. While the majority of these studies were negative[92-96], one study reported VZV positivity by PCR in 26% of TABs with histological evidence of GCA as compared to 0% of controls. This study found no correlation between PCR and immunohistochemistry for VZV (IHC)[97].

In 2013, Nagel et al. [98] reported 5 patients with multifocal VZV vasculopathy involving the temporal artery that mimicked GCA. IHC of temporal artery sections using an anti-VZV antibody was positive in 5/21 TABs from patients with clinically suspected GCA with negative histopathology, suggesting clinical overlap between GCA and VZV vasculopathy. Subsequently, the group reported positive staining for VZV gE antigen by IHC in 74% (61/82) of GCA-positive TABs as compared to 8% of normal TABs, with staining observed in all three layers of the vascular musculature as well as adjacent skeletal muscle. VZV DNA was detected in 40% of VZV antigen-positive TABs (18/45) vs 1 control (8%)[99]. In a subsequent study, the authors found VZV IHC positivity in 73% of GCA-positive TABs, 64% of negative TABs in patients where GCA was suspected on clinical grounds, and 22% of normal temporal arteries from age-matched individuals (removed post mortem)[100]. When blinded to the diagnosis, VZV antigen in 100% (3/3) of GCA-positive TABs vs 67% (4/6) of GCA-negative TABs[101]. Similarly, the same group identified VZV antigen in 100% of patients with granulomatous aortitis and 28% of normal controls at autopsy[102]. Based on these results, the authors suggested VZV as a trigger of GCA and proposed the study of antivirals for the treatment of GCA[103].

The reported high prevalence of VZV positivity by IHC and PCR is awaiting confirmation by other groups. Buckingham et al. [104], using the same anti-VZV gE antibody for IHC, found positive staining in only 12% of TABs from patients with GCA and 0% of biopsy-negative individuals who presented with symptoms compatible with GCA. Notably, unspecific staining using anti-VZV gE was observed in the context of arterial calcifications (a common feature on TAB), on erythrocytes, and in extra-arterial skeletal muscle. Interestingly, one patient with positive staining for VZV antigen in this study was diagnosed with zoster ophthalmicus just 3 weeks prior to symptoms onset[104]. Procop et al. [105] did not detect VZV DNA in 11 temporal arteries (5 GCA, 6 controls) or 31 thoracic aortas (8 GCA, 15 non-vasculitis controls) using two different PCR approaches. In an Italian study of 79 TABs, VZV DNA was not detected in either fixed or frozen tissue, and IHC using anti-VZV gE IgG1 was positive in only one biopsy with GCA and in no controls[106].

In an epidemiological study of US administrative data sets, complicated herpes zoster was associated with an increased risk of GCA, with zoster preceding GCA a small number of cases (3.1-6%)[107]. This is consistent with prior data from the United Kingdom which showed a relative risk increase for herpes zoster in GCA[108]. Studying peripheral blood T

cells in patients with newly diagnosed GCA vs inflammatory and non-inflammatory disease controls of similar age, Bigler et al. [109] found no increase in VZV-specific T cell reactivity by interferon- γ immunospot assay in GCA. While the striking discrepancies in VZV positivity observed by IHC and nucleic acid amplification between groups are difficult to reconcile, the literature may suggest that evidence of VZV can be found in a small subset of patients who were (clinically and histopathologically) classified as GCA. Whether this suggests an active role of VZV in the etiopathogenesis of GCA or simply highlights the inherent difficulty of delineating GCA from its viral mimics in clinical practice remains uncertain.

Kawasaki Disease

Kawasaki disease (KD) is a medium-vessel vasculitis of unknown etiology that predominantly affects children, with highest incidence rates reported in Japan, Korea, and Taiwan[110]. The epidemiology of KD is unique and characterized by large epidemic (Japan) and non-epidemic interannual fluctuations in incidence (Japan, San Diego, USA). These epidemiological patterns have been linked to wind currents originating in central Asia, suggesting wind-borne pathogens as potential triggers of the disease in susceptible individuals[111,112]. The nature of the putative infectious agent is unknown, but bacteria, fungi, and viruses have been discussed. Culture-based studies have found decrease of *Lactobacillus* and presence of HSP60-producing Gram-negative bacterial (*Acinetobacter*, *Enterobacter*, *Neisseria*, and *Veillonella*) and Gram-positive cocci (*Streptococcus* and *Staphylococcus*) in active KD[113,114]. Kinumaki et al. [115] analyzed longitudinal variations in the intestinal microbiota of 28 patients with KD. Several genera were highly increased in the acute versus non-acute phases of KD including *Rothia* and *Staphylococcus*, but this was only observed in a subset of KD patients. Nucleic acid signatures with similarity to 5 *Streptococcus* spp. of the mitis group (*S pneumoniae*, *pseudopneumoniae*, *oralis*, *gordonii*, and *sanguinis*) were found increased in the gut during acute KD in most patients (although with substantial variability), suggesting a potential role for streptococcal species in KD. The microbial composition in the non-acute phase of KD showed increases in relative abundance of genera *Ruminococcus*, *Roseburia* and *Faecalibacterium*[115]. The mechanism by which specific bacterial species may contribute to KD pathogenesis remains speculative, but immune activation via superantigens produced by *Streptococcus* and *Staphylococcus* spp. has been hypothesized to play a role[116].

A role of aerosolized *Candida* toxins disseminated via tropospheric winds originating in northeastern China in the development of KD in Japan, San Diego, and Hawaii have been proposed[112]. Interestingly, the water-soluble extracellular polysaccharide fraction of *Candida albicans* can induce a coronary arteritis in mice[117,118].

ANCA-Associated Vasculitis

A microbial contribution to GPA has long been hypothesized due to the destructive granulomatous inflammation that can affect the upper and lower respiratory tract in this subtype of ANCA-associated vasculitis (AAV). Not surprisingly, several studies have focused on the composition of the nasal and upper respiratory microbiome in patients with

GPA. Preceding culture-independent studies, *Staphylococcus aureus* has repeatedly been implicated in the pathogenesis of GPA[119]. Nasal carriage of *S aureus* by bacterial culture and detection of staphylococcal superantigen was found to be increased in GPA and has been associated with increased risk of disease relapse[120-122]. The benefit of antibiotic treatment with trimethoprim/sulfamethoxazole in preventing relapses in localized disease were thus hypothesized to be mediated by reducing *S aureus* in the upper airways [123-125]. Mechanistic understanding of the association of *S aureus* and GPA remain speculative. While immunosuppressive therapy, local inflammation, and frequent hospitalizations may all account for these findings, dysregulation of epithelial nasal barrier function and impairment of local antimicrobial immune responses have been identified in patients with GPA[126-130]. Interestingly, several leukocidins and hemolysins, *S aureus* pore-forming toxins with activity against neutrophils, were identified in almost all isolates of *S aureus* from patients with GPA, with *luk* (*lukX* and *lukY*) genes being most frequently detected in proteinase 3(PR3)-ANCA isolates as compared to myeloperoxidase(MPO)-ANCA and healthy control group isolates, suggesting a possible role for leukocidins in PR3-ANCA-AAV[129]. Pore-forming toxins from *S aureus* have been shown to induce leukotoxic hypercitrullination (LTH), a form of neutrophil cell death that mimics NETosis and is associated with the extracellular release of neutrophils protein cargo[13,34]. Whether this inflammatory context is sufficient to induce autoantibody responses to cytoplasmic neutrophil proteins PR3 and MPO in a susceptible host is not known.

Exploring the composition of the GPA microbiome, Rhee et al. [131] studied microbial DNA isolated from nasal swabs of 60 patients with GPA and 41 healthy controls by 16S rRNA sequencing. The microbial composition in GPA was significantly different than in normal controls, with lower relative abundance of *P acnes* and *Staphylococcus epidermidis* in GPA. *S aureus* was among the most abundant species identified, but no significant differences in relative abundance between GPA and health were observed. Interestingly, the use of non-glucocorticoid immunosuppression was associated with normalization of the nasal microbiome, with more dysbiosis and lower abundance of *P acnes*, *P granulosum*, and *S epidermidis* primarily observed in GPA patients not on immunosuppressive therapy. *Malasseziales* was the most abundant fungal taxa in the nasal cavity. When compared to GPA in remission or healthy controls, active disease was associated with a lower abundance of *Malasseziales*[131].

In a similar study in the United Kingdom, Wagner et al. [132] identified a higher frequency of nasal carriage of *S aureus* in patients with active versus inactive GPA by culture. Using bacterial 16S rRNA sequencing, active GPA similarly had a higher abundance of *S aureus*, while healthy controls demonstrated more *S epidermidis*. *Staphylococcus pseudintermedius*, a pathogen of cats and dogs, showed an abundance of 13% among *Staphylococcus* spp. in this study. *S aureus* abundance did not predict risk of relapse[132]. In a German study of the nasal microbiome in GPA, bacterial taxa and microbial richness were decreased in GPA compared to RA. *Propionibacteriaceae* and *Staphylococcaceae* showed lower abundance on the family level in GPA, but almost identical changes were observed in RA, suggesting that systemic inflammation or treatment effects rather than disease-specific factors may be driving these changes. *S aureus* presence by qPCR was significantly more abundant in GPA

compared with RA or healthy subjects. Active ENT disease was associated with increases of abundance of *Streptococcaceae* and *Planococcaceae* compared to inactive disease[133].

While most of the studies have explored the nasal microbiome in GPA, there is paucity of data on the microbial composition of other mucosal surfaces in AAV. Preliminary data reported by Najem et al. [134] suggest that the gut microbiome is altered in patients with active AAV, with normalization of microbial composition observed in disease remission.

Other Vasculitides

While an infectious etiology of Takayasu arteritis (TAK) has been hypothesized, no comprehensive data on the microbiome in TAK has been reported. Preliminary data suggests alterations of the “blood microbiome” (sequencing of the 16S rRNA gene blood bacterial DNA) in TAK as compared to healthy controls as well as on TAK with active vs inactive disease[135].

Behçet’s disease (BD) is a variable-vessel vasculitis with autoinflammatory features characterized by recurrent oral and genital ulcers, mucocutaneous lesions, and ocular inflammation, among other organ system involvement. The microbiome in BD has been explored in several studies. Coit et al. [136] analyzed saliva from 31 BD patients and 15 healthy controls using sequencing of the 16S rRNA V4 region. *Haemophilus parainfluenzae* was the most enriched species in BD, while *Alloprevotella rava* was decreased. These differences were not explained by immunosuppressive treatments or genetic risk alleles. Microbial diversity was overall decreased in BD versus controls. Seoudi et al. [137] also studied salivary and oral mucosal microbial DNA, both collected from ulcers and non-lesional mucosa, in 54 BD samples, 8 aphthous stomatitis samples, and 25 healthy controls. Non-ulcer mucosal sites in BD were characterized by increased colonization with *Rothia dentocariosa*, while ulcers showed increased colonization with *Streptococcus salivarius* and *Streptococcus sanguinis* compared to aphthous stomatitis and healthy controls, respectively. Shimizu et al. [138] analyzed changes in fecal microbial composition in 12 patients with BD as compared to 12 normal controls. *Bifidobacterium* and *Eggerthella* were significantly increased, and *Megamonas* and *Prevotella* decreased in BD compared to healthy control. Another study in 22 patients with BD showed relative depletion in the genera *Roseburia* and *Subdoligranulum* in BD as compared to health. Dysbiosis in BD was associated with decrease of butyrate which can promote differentiation of T regulatory cells[139].

IgA vasculitis (Henoch-Schönlein Purpura, HSP) is preceded by respiratory tract infection in the majority of children, suggesting infectious agents in disease etiology[140]. In a recent Chinese study of 98 patients with HSP and 66 healthy controls, 16S rRNA sequencing on oral swabs demonstrated higher microbial diversity in HSP as compared to controls. *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were identified as the dominant phyla in HSP, with 21 bacterial taxonomic clades showing statistical differences between HSP and controls[141]. When examining the fecal microbial composition, HSP was characterized by dysbiosis, exhibiting lower microbial diversity and richness when compared to healthy children. Genera *Dialister*, *Roseburia*, and *Parasutterella* were decreased in children with HSP; the relative abundance of *Parabacteroides* and *Enterococcus* was increased[142].

Summary

Microbiota-immune cell interactions can shape host adaptive immune responses, and microbial contributions to disease initiation and propagation in rheumatic disease are plausible and likely. Studies of the human skin, oral, subgingival, intestinal, nasal, lung, and vascular microbiome suggest that dysbiosis is a common feature across connective tissues diseases and the primary vasculitides. Significant heterogeneity in the observed microbial composition exists across reported studies which may reflect differences in patient population, disease phenotype and activity, treatment, or study design. Efforts in standardization of workflow and methodology are needed to maximize comparability and validity of microbiome research in the study of systemic autoimmune diseases. Integrating descriptive studies with hypothesis-driven research will be critical in identifying plausible microbial targets in the treatment of preclinical and established rheumatic disease.

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Practice points

- Microbiota co-evolved with humans and can shape adaptive immune responses, thereby propagating or ameliorating inflammation and autoimmunity.
- The periodontal pathogens *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* may drive autoantigen citrullination in RA, thereby initiating the ACPA response.
- The gut pathobiont *Prevotella copri* is enriched in new-onset RA and preclinical RA, and may act in RA through inducing Th17 cells and/or molecular mimicry.
- Bacterial species expressing orthologs of human Ro60 may induce anti-Ro60 autoantibody in SLE and SS through cross-reactivity.
- Aerosolized pathobionts could explain seasonal variations and epidemic occurrence of KD in Japan and the US West Coast.
- VZV vasculopathy may mimic GCA, and the diagnosis should be considered in patients with GCA symptoms that present with stroke or recent history of herpes zoster. A primary role for VZV in GCA remains controversial.

Research agenda

- Standardization of workflows in sample collection, nucleic acid extraction, sequencing, data analysis, and reporting are needed to improve reproducibility and comparability in microbiome research.
- Integration of descriptive studies with research informed by immunopathogenesis
- Identification of approaches to target candidate pathogens with high mechanistic plausibility in preclinical patients at risk