Microbiome and mucosal inflammation as extra-articular triggers for rheumatoid arthritis and autoimmunity

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Abstract

\textbf{Purpose of Review—} Despite progress towards understanding the molecular pathogenesis of Rheumatoid Arthritis (RA), its etiology remains elusive. Genes are important but rather insufficient to explain the majority of RA cases. This review describes novel data supporting the microbiome and its interactions with the human host as potential environmental factors in RA pathogenesis.

\textbf{Recent Findings—} Animal models of inflammatory arthritis have shown that the presence of bacteria in mucosal surfaces is sufficient to alter local and systemic host immune responses and elicit joint inflammation. Human RA studies have focused on three mucosal sites: the gut, the gingival, and the respiratory tree. The oral microbiome, and specifically \textit{Porphyromonas gingivalis} (\textit{P. gingivalis}), has long been implicated. Novel sequencing technologies have allowed investigations into the role of the gut microbiome in the development of autoimmune arthritis. Most recently, the pulmonary parenchyma has also been described as yet another possible mucosal site of initiation of autoimmunity in RA.

\textbf{Summary—} Emerging data implicates the microbiome in RA pathogenesis. Mucosal sites exposed to a high load of bacterial antigens - such as the periodontium, lung, and gut - may represent the initial site of autoimmune generation. If validated, these findings could lead to the discovery of potential biomarkers and therapeutic approaches in the pre-clinical and clinical phases of RA.

\textbf{Keywords} 
Rheumatoid; Arthritis; Microbiome; Peridontal; Porphyromonas

Introduction

The notion that the human genome and its encoded machinery are sufficient for our evolutionary success has recently been questioned. In fact, humans are vastly overshadowed...
by their bacterial companions. Literally trillions of bacterial organisms form unique ecological niches in almost all body surfaces. From the moment we are born, humans are populated with a complex, constantly changing community of bacteria that both helps shape the mucosal immune system and provides essential nutrients [1, 2]. Despite their overwhelming antigenic load, most of these microorganisms have long been ignored as potential determinants in the pathogenesis of human disease. Novel technological tools such as culture-independent 16S ribosomal RNA pyrosequencing, advances in computational biology, and initiatives such as the NIH Human Microbiome Project (HMP) have all reenergized this field of study [3]. Microbiome is the term utilized to describe the sum of ecological bacterial communities (and their genes) that populate human skin, oral cavity, airways, gastrointestinal tract (GI), and genitourinary tract (GU). These bacteria and their genomes are classically identified as commensal, symbiotic, or pathogenic; however, the utility of these labels has grown less clear and the interactions between host and bacteria are becoming increasingly complex.

To date, a nearly complete catalogue of the diverse microbial communities in the human body has been revealed [4, 5**]. The largest bacterial burden lies in the intestines, where nearly three pounds of bacteria and over three million bacterial genes outnumber the human host genome 100 times over [6]. The two predominant microbial phyla of the human gut are the Firmicutes, which includes the important Clostridia class, and the Bacteroidetes. Also present but less pervasive are the phyla Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia [7]. Interestingly, although debated, studies have identified three core human microbial enterotypes [8] that cluster subjects according to the relative abundance of the microbial genera Bacteroides, Prevotella, and Ruminococcus [9**].

Despite the advent of genome-wide association studies (GWAS) and intense research into both MHC and non-MHC genetic polymorphisms, the etiologies of most rheumatic diseases remain elusive. Multiple genetic studies have shaped our understanding of RA susceptibility (i.e. shared epitope hypothesis) [10] and continue to elucidate pathways and function [11]; however, causal evidence is lacking, as illustrated by a relatively low concordance rate in monozygotic twins. Thus, much focus has shifted to the role of environmental factors and gene-environmental interaction in the pathogenesis of rheumatic diseases. This is particularly true for RA because evidence for autoimmunity in the form of circulating anti-cyclic citrullinated peptide (CCP) antibodies and/or rheumatoid factor (RF) can be found up to a decade before disease onset [12]. Interestingly, both antibody titers and epitope specificity rise just a few months before synovitis is apparent, and circulating pro-inflammatory cytokines are also elevated during this pre-clinical phase [13]. Moreover, synovial biopsies and joint MRIs of asymptomatic individuals with circulating anti-CCP fail to demonstrate signs of tissue damage [14*]. Taken together, these data are highly relevant because they suggest that autoimmunity in RA has an extra-articular origin (i.e. outside-in process) and that a “second hit” is necessary for arthritis development. Among possible non-genetic triggers, the microbiome has been identified as a possible inciting factor. This review will focus on the recent evidence implicating the gut, oral, and lung microbiomes as en(’in’)vironmental factors in autoimmune arthritis (Figure).
Microbiome and Murine models of inflammatory arthritis

The notion that gut microorganisms can modulate extra-intestinal autoimmunity is not novel. As the gut represents the largest reservoir from which the immune system actively samples antigens, it seems plausible that the microbial make-up of a susceptible individual could influence the initiation, progression, and/or intensity of local and systemic autoimmune disease. Seminal work in animal models conducted more than three decades ago have established a relationship between the development of inflammatory arthritis and the presence or absence of certain bacterial genera (Table). Rat models of adjuvant-induced and streptococcal cell wall-induced arthritis show protection against the development of arthritis in the presence of mucosal microbes. In both studies, germ-free reared rats showed increased vulnerability to arthritis [15, 16]. In contrast, a germ-free environment (sterile cages devoid of microorganisms) is protective against the development of arthritis in the spontaneous spondyloarthropathy model of HLA-B27 transgenic rats [17]. This may be explained by recent work showing that misfolded HLA-B27 in LPS-stimulated macrophages resulted in robust increases in proinflammatory cytokines IL-23 and IL-17, along with intestinal inflammation [18]. Additionally, HLA-B27 transgenic rats lack certain populations of dendritic cells important in maintaining tolerance to self-antigen in their mesenteric lymph nodes [19]. Similarly, both the IL-1 receptor antagonist knockout (IL-1RA −/−) and the K/BxN mouse models of arthritis remain healthy in germ-free environments. Gavaging these mice with *Lactobacillus* and segmented filamentous bacteria (SFB) respectively, is sufficient for development of autoimmunity and inflammatory arthritis via induction of a robust TH17 response [20, 21]. The ZAP-70 single-point mutation mouse model, SKG, also develops joint inflammation when reared in conventional cages. In these mice however, lung-residing fungal organisms appear to be responsible, as SKG mice harbor a larger respiratory fungal load and inflammatory arthritis can be induced by injection of beta-glucans, a component of fungal cell-wall [22]. Most recently, inflammatory arthritis was induced in rats by introducing oral antigens in the setting of mucosal barrier dysfunction [23]. Taken together, these studies reveal a role for the microbiome in various susceptible animal models and validate a mechanistic relationship between microbes, mucosal immunity, and joint inflammation.

A recent experiment investigated the microbiome of arthritis-susceptible *0401 transgenic mice and arthritis-resistant *0402 transgenic mice [24**]. The *0401 mice microbiome was dominated by *Clostridium* bacteria and bacterial diversity was not influenced by age or sex. In contrast, the resistant *0402 mice were enriched by the *Porphyromonadaceae* and *Bifidobacteria* families, and they displayed strong influences of age and sex. Additionally, the susceptible mice demonstrated increased mucosal permeability and an altered TH17 gene transcriptomic profile. These findings suggest the possibility that host genetics may indeed represent a predetermined factor for harboring a unique microbiome profile.

Gut dysbiosis and RA

Linking intestinal dysbiosis (i.e. alteration of the gut microbiome homeostasis) to RA pathogenesis is far from a novel concept. In fact, the *toxemic factor* hypothesis was originally formulated at the turn of the twentieth century. It proposed that the pathological
abundance of gram negative bacteria in the intestinal canal led to an increase in toxic metabolites that entered the circulation and ultimately promoted joint inflammation. In fact, therapeutic regimens that theoretically target the intestinal flora and the gut-joint axis have been in use since the 1940s (Figure). Several of them even became disease-modifying antirheumatic drugs (DMARDs), such as sulfasalazine and minocycline. Since then, however, there have been relatively few studies directly evaluating the gut microbiota in human RA.

In one study, low-throughput 16S hybridization technique (only 8 probes included) was used to evaluate the fecal microbiota of patients with newly diagnosed RA [25]. RA patients showed overall fewer bacterial counts when compared to patients with fibromyalgia. A second study investigated the composition of fecal lactobacillus communities in early RA patients. Using specific lactobacillus primers, this study revealed a higher diversity of the genera in early disease [26]. Specifically, there were increased levels of L. salivarius, L. iners, and L. ruminis in the RA group compared to controls, and the presence of L. mucosae was unique to RA patients.

We have recently observed that the majority of new-onset, DMARD-naïve RA patients carry high levels of Prevotella copri, a unique species that defines the microbial genome of these patients (manuscript in press, eLife Journal 2013). Gut Prevotella, a gram-negative anaerobe, appears to be relevant in many inflammatory and autoimmune conditions. In addition to being considered one of the three recently described enterotypes, Prevotella has also been found in patients with inflammatory bowel disease and was sufficient to induce colitis in an inflammasome knock out mouse model (i.e. NLRP6 pathway) [27]. Most recently, Koeth et al. demonstrated that high abundance of gut Prevotella in healthy subjects correlated with increased plasma levels of trimethylamine N-oxide (TMAO), a substance predictive of cardiovascular events in humans. Metabolism by intestinal microbiota of dietary L-carnitine, a compound abundant in red meat, also produced TMAO and accelerated atherosclerosis in mice, but this did not occur if the intestinal microbiota was concurrently suppressed [28*]. These findings are relevant because they may partially help explain the higher incidence of cardiovascular disease in some RA patients.

Despite the recent animal and human evidence implicating the intestinal microbiome in RA development, it remains uncertain whether these states of gut dysbiosis represent a causal-etiologic factor or rather a secondary effect of local and systemic inflammation. Further work is required to better understand the enzymatic and metabolic effects underlying the changes in gut bacterial communities in RA patients.

Evidence for the RA-periodontal disease association

Periodontal disease (PD) is a common polybacterial infection of the gingival tissue [29]. RA and PD are known to share pathogenic mechanisms and immunologic pathways of bone and tissue destruction. In both diseases, adaptive and innate immune cells promote secretion of pro-inflammatory cytokines (i.e. IL-1, IL-6, and TNF) and other degrading substances such as matrix metalloproteinases and reactive oxygen species [30–31]. Moreover, although proposed more than a century ago, an epidemiologic association between RA and PD has...
recently been validated. A number of studies have now demonstrated an increased incidence and severity of PD in RA [32, 33*, 34]. A recent population-based effort reinforced an association, albeit rather weak, between a prior history of PD and new onset RA, suggesting a causal role for PD in RA pathogenesis [35]. These findings are not universal, however, and a recent study utilizing patient-reported data failed to demonstrate the association [36]. Additional longitudinal studies are therefore needed to establish a role for PD in RA pathogenesis. Intriguingly, however, treating PD appears to improve RA severity and concomitantly decrease pro-inflammatory biomarkers of disease activity such as CRP, ESR, TNF, and IL-1beta [37–38]. RA patients with active PD are also less responsive to anti-TNF-α inhibition, indicating a potential role for the co-treatment of PD in TNF refractory patients [39].

Much research has focused on the specific bacteria associated with PD. The healthy oral microbiome is second in diversity only to the gut and is dominated by the bacterial phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes, and Fusobacteria [40]. PD has traditionally been linked to the “red-complex” bacteria, which include P. gingivalis, Treponema denticola, and Tannerella forsythia [41]. New technologies, particularly the aforementioned 16S pyrosequencing, have confirmed these results. Meanwhile, other taxa have also been associated with both PD development and other aggravating environmental risk factors such as cigarette smoking [42 – 43*].

Despite the established knowledge that PD is actually a polymicrobial disorder, RA research has focused mostly on P. gingivalis. This bacterium is presumably the only known prokaryote with a gene encoding for peptidylarginine deiminase (PAD), an enzyme that converts arginine residues into citrulline [44 – 45]. The citrullination of mucosal protein-peptides such as vimentin, keratin, and α-enolase reportedly generates neo-epitopes that can then foster loss of immune tolerance and eventually the production of anti-citrullinated protein antibodies (ACPAs). ACPAs are the most specific biomarkers for RA to date, are found in 70–80% of patients, and are known to confer worse prognosis [46 – 47]. The prevalent hypothesis posits that Pg (and the humoral response to it) represents an antigenic source for the creation of ACPAs and subsequent progression to RA [30]. This was further validated by studies showing that P. gingivalis-derived PAD was capable of citrullinating the human fibrinogen and α-enolase isoforms [48], and that human α-enolase could also induce autoimmunity and inflammatory arthritis in DR4-transgenic mice [49]. Additionally, treatment of PD can decrease the levels of circulating ACPAs and anti-P. gingivalis antibodies [50].

Exposure to P. gingivalis has been indirectly assessed serologically in both clinical RA and in subjects considered to be at-risk for RA development (i.e., first-degree relatives, Native Americans or those with ACPA positivity without synovitis). Anti- P. gingivalis antibodies correlated with the presence of ACPAs in both groups when compared to controls [51, 52*, 53]. Utilizing pyrosequencing to assess the oral microbiome of patients with new onset, DMARD-naive RA, we showed a direct presence of P. gingivalis in slightly more than half the cohort. The relative abundance of P. gingivalis, although not statistically significant, was two-fold higher in patients with new-onset RA compared to non-RA controls. Most microbial differences, however, could be attributed to the presence of concomitant advanced
PD [54**]. The presence of other taxa, such as *Anaeroglobus geminatus* and *Prevotella/Leptotrichia* were intriguingly associated with ACPA seropositivity and clinical RA. Overall, the cumulative data argues for a potential involvement of PD and *P. gingivalis* in the development of autoimmunity and clinical RA. Additional population and basic investigations are required to elucidate this complex picture involving altered bacterial diversity in periodontal tissues and the consequent microbe-host interactions in the form of local and systemic inflammatory responses.

**Airway inflammation and RA – a role for the microbiome?**

The human airways constitute a vast mucosal surface constantly challenged by a high microbial-derived antigenic diversity. Similarly to the oral cavity and the intestinal tract, the respiratory mucosa is home to its own characteristic microbiome [55–57]. Interestingly, cigarette smoking (arguably the best studied environmental risk factor for RA) is not definitively responsible for producing an altered lung microbiome [55–56, 58]. Although studies found no association between smoking and modified bacterial diversity, Segal et al. [58] did note a correlation between increased airway inflammation and transposition of traditionally supraglottic bacteria into the lungs, particularly with *Prevotella* and *Porphyromonas*.

Although there are no reports directly evaluating the lung microbiome in patients with RA, several studies have suggested a possible role in pathogenesis. Chief among those is the evidence that the lung is a site for local citrullination. Cigarette smoking has been shown to generate increased PAD expression and protein citrullination in bronchoalveolar lavage (BAL) cells of smokers without arthritis [59], and a subset of patients with lung disease in the absence of existing RA test positive for plasma anti-CCP antibodies. Moreover, PAD polymorphisms, such as PADI4, have also been shown to predispose male smokers to RA [60]. This association appears to be cigarette smoking specific since non-smoking particulate matter (i.e. a measure of air pollution) was not associated with increased risk of progression to RA in asymptomatic seropositive patients [61].

Recent studies examined early RA patients and at-risk individuals (i.e. autoantibody positive) for signs of airway abnormalities using high resolution CT [62**]. Signs of inflammation, such as bronchial wall thickening, bronchiectasis, and centrilobular opacities, were common in at-risk subjects and were similar to airway abnormalities seen in patients with early RA. In a separate report, anti-CCP antibodies and RF were found in induced sputa of several patients from the same cohort [63**]. Interestingly, enrichment of the lung microbiome with supraglottic taxa (including oral *Porphyromonas* and *Prevotella*) was associated with increased airway inflammation. Taken together, these data suggest that the lung may be an early site of autoimmune-related injury and/or potentially a site of generation of RA-related autoimmunity, perhaps due to the presence of periodontal tissue-derived pro-inflammatory microbiota. These data suggests that the lungs participate in the citrullination of proteins and consequent generation of autoimmunity. The potential role of PD-associated bacteria in inciting those inflammatory responses, perhaps due to microaspiration, has only recently been described and requires further investigation.
Conclusions

A growing body of evidence linking the microbiome with RA pathogenesis is now becoming available. Animal models set a solid foundation for linking bacterial antigenicity to the generation of inflammatory arthritis. However, the application of these studies to human populations is complex, and further investigations into bacterial-host mucosal interactions are needed. The microbiome is extensive and multiple human body sites are home to a wide diversity of bacterial populations. The PD-associated bacteria are the most extensively studied. Strong evidence confirms the ability of Pg to citrullinate host proteins, but the relevance of this one bacterium is less obvious when assessed in isolation. A polymicrobial effect in periodontal inflammation and RA pathogenesis seems more likely. The gut carries the largest bacterial burden and is the most directly linked to RA when evaluating animal studies. To date, only a handful of human studies have investigated the gut microbiome in RA patients. Results are encouraging but mechanistic insights to address causality are still lacking. The notion that the respiratory mucosa could play a role in RA pathogenesis is relatively new. Advanced imaging techniques indicate that airway inflammation and autoimmunity predate joint disease, as they are often present in the pre-clinical phases of RA. Taken together, in vitro, animal, and human data suggest that host-bacterial interactions may play a role in autoimmunity initiation.

One possibility is that bacteria sharing similar pro-inflammatory properties may serve as triggering factors in various mucosal sites, particularly in genetically predisposed individuals. “Pan-microbiome” studies will need to be conducted as they may hold an important key to a deeper understanding of RA pathogenesis. The validation of recent findings while studying the microbiome in the various phases of RA (and in different at-risk populations) will be of the utmost importance. Synergistic in vivo and in vitro work will also be required to advance our knowledge in this field, which is promising for its discovery potential in biomarker identification and new therapeutic targets.

References


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63. Willis VC, Demoruelle MK, Derber LA, Chartier-Logan CJ, Parish MC, Pedraza IF, et al. Sputa autoantibodies in patients with established rheumatoid arthritis and subjects at-risk for future clinically apparent disease. Arthritis & Rheumatism. 2013 n/a–n/a. This study shows that patients at-risk for RA development have measurable auto-antibodies in sputa.
Key Points

- Animal models of inflammatory arthritis demonstrate the importance of the microbiome in the development of local and systemic autoimmunity.
- There is validated evidence that the autoimmune process in RA begins many years before the emergence of clinical synovitis. The source of autoantibodies such as RF and anti-CCP appear to be extra-articular in nature since seropositive individuals without RA have normal synovial tissue.
- Mucosal surfaces constantly exposed to bacterial antigens are being studied as possible sites of autoimmunity initiation.
- Peridontal disease is strongly linked to RA, and an altered oral microbiome has been identified in patients with early RA. *P. gingivalis* and its enzymatic machinery for peptide citrullination appears to play a role in pathogenesis.
- The community bacterial composition in the gut is altered in mouse models of inflammatory arthritis and in human patients with RA.
- Airway inflammation and autoimmunity (i.e. anti-CCP and RF antibodies) appear to be present even at the pre-clinical phase of disease. Studies of the RA lung microbiome are currently underway.
Figure.
Potential sites of microbiome involvement and related risk factors in the pathogenesis of RA. A. Periodontal disease is linked to increased RA prevalence and disease severity. The oral microbiome, specifically \textit{P. gingivalis} through PAD activity, has been implicated. B. The lung mucosa has increased PAD activity and airway inflammation is more prevalent in both seropositive, at-risk subjects (i.e. anti-CCP positive) and RA patients. C. An altered gut microbiome has been identified in multiple animal models and in human RA.
TABLE

Murine models of inflammatory arthritis, associated gut microbiota and potential immunological mechanisms involved. Four of the most recent animal models of inflammatory arthritis. The presence of bacteria seems to mainly act via inducing local and systemic Th17 cell response. Decreased production of Treg cells and increased gut permeability are also implicated.

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>Identified taxa</th>
<th>Proposed Immune Mechanism</th>
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<tbody>
<tr>
<td>HLA-B27 transgenic rat</td>
<td>- Bacteroides</td>
<td>HLA-B27 misfolding; increased IL-23 and IL-17</td>
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<tr>
<td>IL-1 receptor antagonist knockout mice (with TLR2/TLR4 double knock out)</td>
<td>- Lactobacillus</td>
<td>TLR2−/− leads to decreased Tregs and increased in IFN-γ production</td>
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<td>TLR4−/− leads to decreased Th17 cells and IL-17 production (less arthritis incidence/severity)</td>
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<td>K/BxN T-cell receptor transgenic mice</td>
<td>- Segmented filamentous bacteria (SFB)</td>
<td>Induction of Th17 cells in the intestinal lamina propria and periphery</td>
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<tr>
<td>Arthritis-susceptible *0401 transgenic mice</td>
<td>- Clostridum</td>
<td>Increased gut mucosal permeability with increased induction of Th17 gene transcripts</td>
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