REVIEW

Multiple hit infection and autoimmunity: the dysbiotic microbiota–ACPA connection in rheumatoid arthritis

Lazaros I. Sakkas and Dimitrios P. Bogdanos

Purpose of review
This review highlights the most recent data obtained in this field and provides clues toward the better understanding of the close interplay between microbiota and host, leading to autoimmune diseases.

Recent findings
A well-described model of microbiota/host interaction of relevance to autoimmunity is that linking anti-citrullinated peptide antibody positive rheumatoid arthritis and alterations of microbiota largely concentrating on *Porphyromonas gingivalis* and more recently of *Aggregatibacter actinomycetemcomitans and Prevotella copri*.

Summary
The perception of the classical link between microbial infection and development of autoimmune disease has evolved to the more recent concept of the connection between the microbiome/dysbiosis and breaking of immunological tolerance.

Keywords
autoantibody, infection, microbiome, rheumatic diseases, rheumatoid arthritis

INTRODUCTION

It is well established that genetic factors, such as HLA-DRB1 shared epitope (HLA-DRB1SE) alleles [1], and environmental factors are involved in the development of rheumatoid arthritis (RA), whereas proinflammatory Th1 cells and B cells and proinflammatory soluble mediators [tumor necrosis factor alpha (TNFα) and interferon gamma, interleukin-6 (IL-6)] are involved in disease pathogenesis [2]. RA is considered an autoimmune disease for the presence of the autoantibodies (autoabs) rheumatoid factor and abs against citrullinated peptides [anti-CCP abs and anti-citrullinated protein autoantibodies (ACPAs)]. Citrullination is a posttranslational modification of proteins caused by peptidyl arginine deiminases (PADs).

The discovery of ACPAs greatly advanced our understanding of RA pathogenesis and put citrullinated antigens as likely pathogenic autoantigens for this disease [3]. ACPAs appear long before, even years before clinical onset of RA [4–6]. Early on, ACPAs target few citrullinated peptides, but their targets increase as the onset of clinical arthritis approaches [5,7] and this expansion of ACPA targets is associated with the appearance of proinflammatory mediators and subclinical inflammation [7]. ACPAs are associated with severe disease and their presence predicts subsequent development of RA in patients with undifferentiated arthritis [3,8]. In fact, ACPA is the strongest predictor of radiographic progression in RA [9]. Moreover, the genetic factor HLA-DRB1SE is associated with ACPA rather than RA [10].

Citrullination could create particular neoantigens that activate T cells, which in turn will provide antigen-specific help to B cells to produce ACPA. Indeed, citrullination increases the affinity of peptide to HLA-DRB1SE alleles [11,12]. However, T cells may recognize PAD, instead of citrullinated

Department of Rheumatology and Clinical Immunology, Faculty of Medicine, School of Health Sciences, University of Thessaly, University General Hospital of Larissa, Larissa, Greece

Correspondence to Dimitrios P. Bogdanos, MD, PhD, Department of Rheumatology and Clinical Immunology, Faculty of Medicine, School of Health Sciences, University of Thessaly, University General Hospital of Larissa, 4110 Larissa, Greece. Tel: +30 241 350 2880;
fax: +30 241 350 1016;
e-mail: bogdanos@med.uth.gr, www.autorheumatology.com

*Lazaros I. Sakkas, Dimitrios P. Bogdanos shared last authorship.

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KEY POINTS

- Autoimmune process as exemplified by ACPA detection in rheumatoid arthritis begins outside joints years before clinical arthritis.
- Mucosal sites, at the interface between environmental factors and host, are most likely sites of immune triggering.
- Oral and gut microbiota are altered in RA.

peptides, and then help B cells to produce abs against citrullinated proteins bound to PAD, as a hapten/carrier case [13▪].

The appearance of ACPAs years before the onset of subclinical arthritis suggests that the disease process may begin outside joints. Mucosal sites are attractive candidates as they are the interface between environmental factors and the host. Mucosal immunity could initiate an immune response that leads to breaking of immune tolerance, driving systemic inflammatory immune response and culminates in clinical arthritis. An integral functional part of mucosa sites is microbiota. Components of microbiota activate specific immune cells and shift the balance between proinflammatory and anti-inflammatory response. For instance, segmented filamentous bacteria, gut commensals, promoted Th17 cells in small intestinal lamina propria and spleen and exacerbated arthritis in K/BxN T-cell receptor transgenic mice living in germ-free environment [14]. On the contrary, *Prevotella histicola (P. histicola)*, a commensal of human gut, has immunoregulatory properties and suppresses proinflammatory cytokines. *P. histicola*, when administered enterally to HLA-DQ8 transgenic mice, increased suppressor intestinal and splenic dendritic cells, greatly decreased proinflammatory Th17 cells, IL-17 and TNFα, increased anti-inflammatory IL-10 and reduced intestinal permeability and severity and incidence of collagen-induced arthritis (CIA) [15]. Two known environmental risk factors for RA, smoking and periodontitis [16–18], are in agreement with this concept of immune initiation at mucosal sites.

ORAL MICROBIOTA

Periodontitis, an example of oral microbiota alteration, dysbiosis, is a risk factor for RA [16–18] and could provide the initial immune response that leads to the breaking of tolerance (Fig. 1): Abs against bacterial proteins, through molecular mimicry, are redirected toward human proteins, and there is growing evidence to support this view. Untreated patients with periodontitis exhibit ACPAs [19]. Gingival grevicular fluid collected from the space between the tooth and gingival mucosa from patients with periodontitis exhibited extensive protein citrullination, mirroring the hypercitrullination in RA joints, whereas it showed minimal citrullination in healthy controls without periodontitis [20]. Schwenzer *et al.* [21] used liquid cromatograph-tantem spectrometry in gingival crevicular fluid and gingival tissue from patients with periodontitis and identified a novel citrullinated peptide of cytokeratin-13 (cCK13). ACPAs against cCK13 were present in 24% of RA patients (specificity 98%) and associated with abs to *Prevotella intermedia*, a causative agent of periodontitis, whereas ACPAs against α-enolase (CEP-1), vimentin and fibrinogen were associated with smoking and HLA-DRB1SE. New-onset untreated RA (NORA) patients had alterations of oral microbiome and a high prevalence of periodontitis [22]. The oral (saliva, dental) microbiome, altered in RA, was partially restored after RA treatment [23].

A causative agent of periodontitis, *Porphyromonas gingivalis (P. gingivalis)*, causes citrullination of bacterial and human proteins [24]. *P. gingivalis* produces gingipains, proteases expressed on the surface of bacterial outer membrane, that cleave proteins at peptidyl arginine, and PAD (PPAD) that preferentially citrullinates C-terminal arginine, thus creating neoantigens. There is evidence of *P. gingivalis* infection years before onset of clinical arthritis. IgG abs to arginine ginginpain B (RgpB), a surrogate marker of past infection with *P. gingivalis,* were detected 10 years before the onset of clinical RA, and their titres increased before the clinical onset of arthritis [25]. Children with ACPA-positive juvenile idiopathic arthritis have high levels of anti-*P. gingivalis* abs compared to controls [26]. In ACPA-positive RA, an interaction between anti-RgpB abs, smoking and HLA-DRB1SE was reported [27]. ACPA to citrullinated α-enolase peptide 1, an immunodominat peptide in RA, showed high homology with α-enolase from *P. gingivalis* and cross-reacted with citrullinated recombinant *P. gingivalis* enolase [28]. *P. gingivalis* inoculation of mice caused PPAD-dependent exacerbation of CIA [29].

Oral inoculation of *P. gingivalis* in HLA-DR1 transgenic mice transiently increased Th17 cells in regional lymph nodes and peripheral blood, induced a massive increase in proinflammatory cytokines, decreased bone density and exacerbated CIA [30]. However, one should take into account the infectious load of *P. gingivalis* inoculation, relative to real-life *P. gingivalis* oral concentrations in patients. *P. gingivalis* can affect inflammation through gut microbiota [31,32].

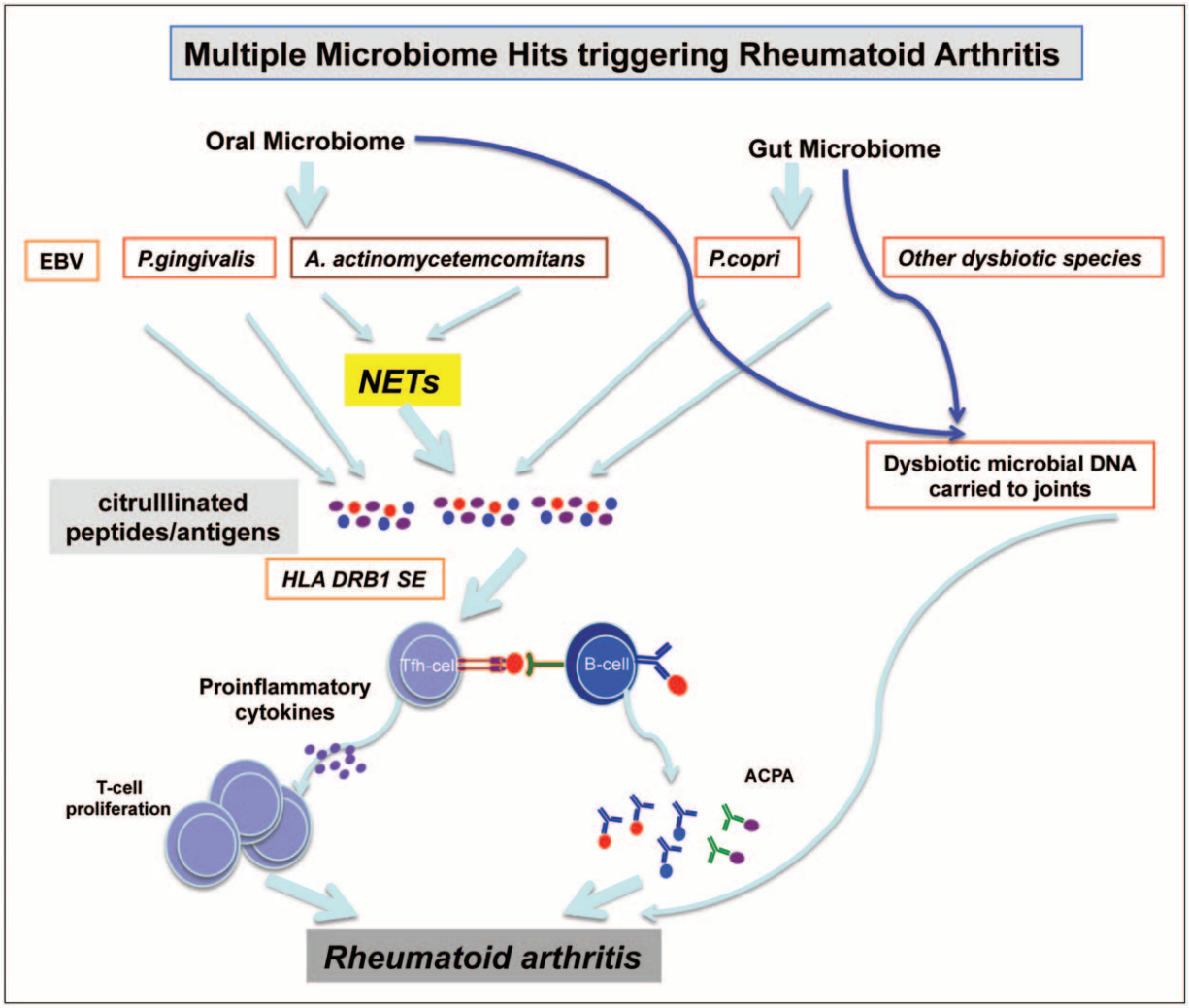

FIGURE 1. Periodontal disease caused by *P. gingivalis* and *A. actinocycetemcomitans* or viral infection with Epstein–Barr virus can lead directly or indirectly through neutrophil extracellular traps (NETs) citrullination of proteins/peptides which in susceptible individuals (HLA DRB1) can lead to the induction of T cells which recognize citrullinated peptides and accelerate an autoreactive immune response which culminates in the development of rheumatoid arthritis. Gut microbiome constituents such as *Prevotella copri* or other species can also initiate an immune response which can also lead to the development of rheumatoid arthritis.

Another causative agent of periodontitis, *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)*, also causes citrullination of human proteins through production of leukotoxin A that forms pores in leukocyte membranes [20,24]. Interestingly, the HLA-DRB1SE is associated with ACPA only in RA patients exposed to *A. actinomycetemcomitans* [20].

Epstein–Barr virus (EBV), which infects epithelial cells and B cells, also causes ACPA production. ACPAs against peptides derived for EBV nuclear antigen (EBNA)1 (PCV1) and EBNA2 (PCV2) cross-react with human citrullinated proteins [33]. Furthermore, ACPAs against PCV1 and PCV2 and ACPAs against histone-4-derived citrullinated peptide (HCP1) and HCP2 appear years before the onset of clinical RA and predict with high-risk ratio (odds ratio = 8–19) subsequent development of RA [25].

GUT MICROBIOTA

The gut microbiota plays a critical role in developing normal immune system [34]. An early study demonstrated that HLA-DR allleles may affect gut microbiota. In transgenic mice carrying the RA susceptible allele HLA-DRB1*0401, the gut microbiome was dominated by *Clostrium*-like species, whereas in transgenic mice carrying the RA resistance allele HLA-DRB1*0402 the gut microbiome was enriched in *Porphyromonadacaeae* and *Bifidobacteria* [35]. Therefore, it is of no surprise that gut dysbiosis has been found in RA (Fig. 2). Dysbiosis in RA

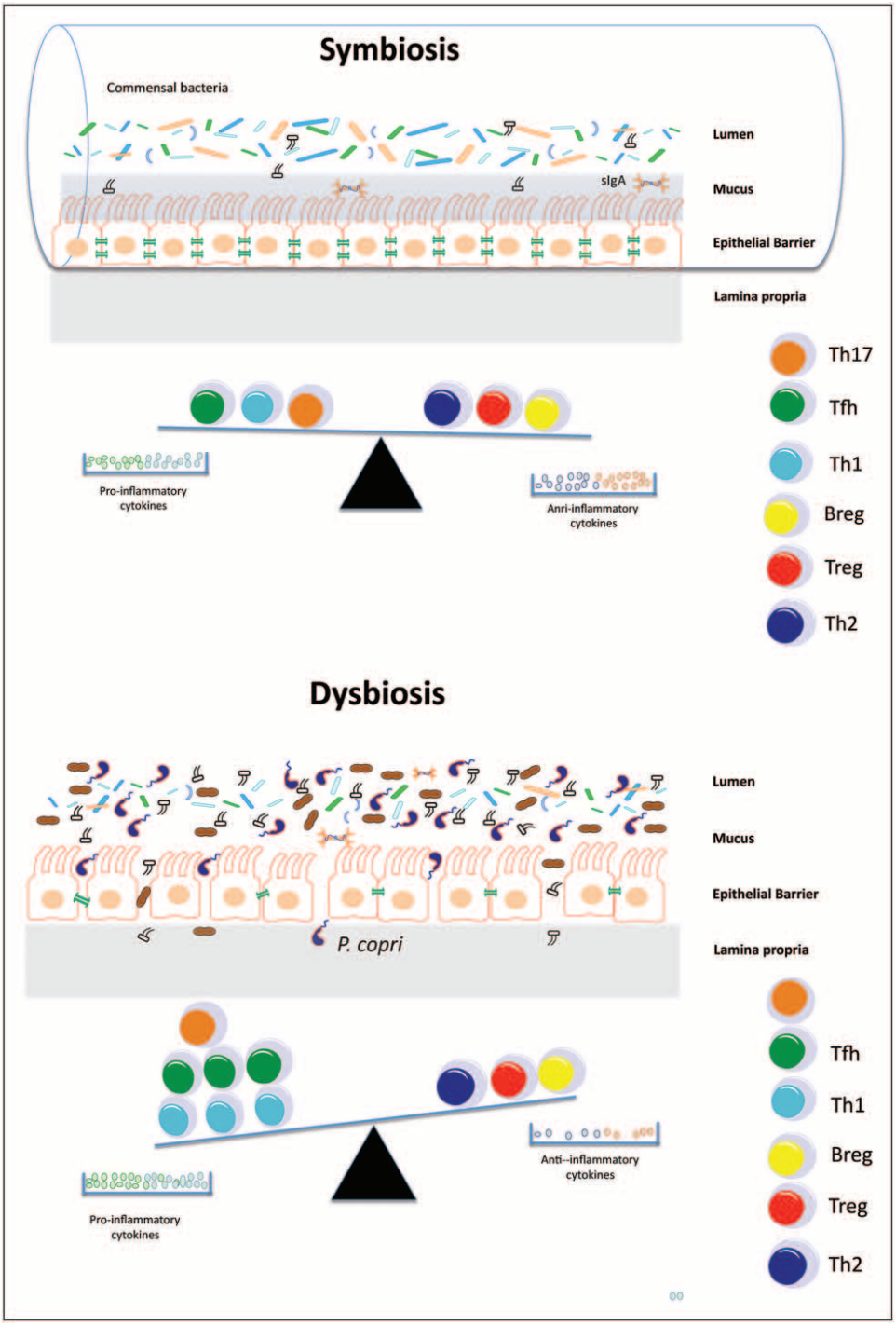

FIGURE 2. (a) When symbiosis, a state characterized by a predominance of commensal bacteria and symbionts, is established, there is a fine balance between proinflammatory and anti-inflammatory cell subsets which prevents from the development of the disease. (b) Such a balance is interrupted when dysbiosis, a state whereby pathogens of the gut microbiome prevail. In symbiosis the intestinal barrier is undamaged and the mucosal homeostasis is maintained due to tolerance to commensal bacteria while in dysbiosis the intestinal barrier is damaged and the protective action of secreted IgA and anti-inflammatory cells (Tregs and Bregs) is lost giving space to proinflammatory mucosal T effector and Th17 cell dominance. Such an imbalance leads to the breaking of immunological tolerance and the perpetuation of autoreactive processes inducing autoimmune disease.

may harbor expansion of rare microbes, such as *Collinsella*. *Collinsella*, which correlated with increased IL-17 production *in vitro* and increased IL-17A in RA patients *in vivo* [36]. Furthermore, *Collinsella* increased gut permeability in HLA-DQ8 transgenic mice [36]. *Prevotella copri* (*P. copri*) was found to be expanded in NORA patients and be correlated with reduction in gut *Bacteriodes* [37].

Reduction of *Bacteriodes* is likely to promote proinflammatoy environment, as polysaccharide A from *Bacteroides fragilis*, a human gut commensal, induced $CD4^{+}$T-cell differentiation into Foxp3+-$TregsproducingIL-10$[38].

P. copri dominated the gut microbiome in early RA patients, whereas SKG mice, colonized with fecal samples (microbiota) from these patients, exhibited increased gut Th17 cells and developed severe arthritis when treated with zymosan, while T cells from the large intestine increased IL-17 production in response to arthritis-related 60S ribosomal protein L23a (RPL23A). Furthermore, RPL23A-responsive T cells, previously cocultured with *Prevotella*-dominated RA feces suspension, induced arthritis in severe combined immunodeficiency mice suggesting that dysbiosis can facilitate inflammation/arthritis by inducing proinflammatory gut immunity [39].

HLA-DR-bound peptides (T-cell epitopes) from RA synovial membrane cells were eluted and analyzed by highly sensitive nanoflow liquid chromatography-tandem mass spectrometry and peptide sequencing in a study. By this approach, Pianta *et al*. [40▪▪] identified two new T-cell autoantigens, one derived from acetylglucosamine-6-sulfatase (GNS) and one derived from filamin A (FLNA). Both peptides were recognized by T cells and B cells. GNS protein appears to be citrullinated *in vivo* as abs against citrullinated GNS were higher than abs against uncitrullinated GNS and the titres of abs against citrullinated GNS correlated with ACPA levels [40▪▪]. Anti-GNS abs and anti-LFNA abs were correlated with abs against *P. copri*, but not with abs against *P. gingivalis*. There was little overlap between NORA patients with anti-*P. copri* abs and NORA patients with anti-*P. gingivalis* abs [41], suggesting that *P. gingivalis* does not allow the growth of *P. copri* and vice versa. Antibodies against GNS and FLNA combined were present in 55% of ACPA-negative NORA patients. There was sequence homology between GNS peptide and peptides from *P. copri*, and *Parabacteriodes* species, particularly aminoacids, predicted to bind HLA-DR. Also, there was sequence homology between FLNA peptide and another peptide from *P. copri*, but no homology between GNS or FLNA and *P. gingivalis*. There was cross-T-cell reactivity between microvial and self-peptides, as strongly suggested by the finding that of the eight RA patients who had T-cell reactivity to GNS peptide, seven had also reactivity to bacterial peptides, and similar analogy was observed with the FLNA peptide. Moreover, the magnitude of T-cell reactivity to GNS or FLNA peptides correlated with that of reactivity to microbial corresponding microbial peptide. Both GNS and FLNA peptides were predicted to bind strongly HLA-DRB1SE and T-cell reactivity to these peptides was more frequent in patients carrying the HLA-DRB1SE [40▪▪].

P. gingivalis can cause arthritis through changes of gut microbiota. Orally administered *P. gingivalis*, but not *P. intermedia*, changed the gut microbiome with decrease in *Bacteriodetes* phylum, increased Th17 cells in mesenteric lymphocytes, increased intestinal permeability, increased bacterial DNA in the liver and aggrevated CIA [31,32,42]. As might have been expected, the gut dysbiosis in RA, was partially restored after RA treatment [23] and this offers some thoughts for new therapeutic strategies in RA.

Gut dysbiosis may trigger autoimmunity by inappropriate posttranslational modification, such as citrullination, ubiqutination, transglutamination and so on [43]. For instance, microbial transgutaminases by cross-linking human proteins can create neoepitopes that are immunogenic [44]. Microbiota is likely to cause arthritis not through Th17 cells but through follicular helper T cells (Tfh cells), which interact with B cells for germinal center formation. In the K/BxN T-cell receptor transgenic mouse model of autoimmune arthritis, deletion of Bcl6, a transcription factor for differentiation and function of Tfh cells, blocked Tfh cell differentiation and the development of arthritis. Tfh cells migrate into distal lymphoid tissues and augment autoab response [45,46]. Microbiota and their metabolites can also exert epigenetic modifications. For instance, the short-chain fatty acids (SCFAs), butyrate and proprionate, produced by gut microbiota from dietary fiber, suppress histone deacetylase and promote generation of Tregs [47]. Also, SCFAs induced gene expression for B-cell differentiation and provide building blocks and energy for antibody production [48].

It is also possible that in dysbiosis, microbial DNA is carried to joints by macrophages. *P. copri* DNA was identified in synovial fluid from early RA patients with IgG anti-*P. copri* antibodies [41]. *P. gingivalis* DNA was also detected in synovial tissue from RA patients. More interestingly, *P. gingivalis* DNA in synovial tissue was detected more frequently in HLA-DRB1∗04-positive than HLA-DRB1∗04-negative RA patients [49]. These findings suggest that molecular mimicry between dysbiotic bacteria and humans may operate as well in RA. Another mechanism could be through an inflammatory milieu. DNA from periodontopathogenic bacteria stimulates macrophage IL-6 and TNFα production [50].

A recent study found that cross-reactivity of ACPAs is much broader than ever imagined. A monoclonal ACPA, obtained from patients with RA by an immunospot array assay, exhibited cross-reactivity

with many citrullinated human, bacterial, fungal, viral proteins and plant proteins present in daily food [51]. If this is confirmed, it puts cross-reactivity into a much more complex perspective.

CITRULLINATED ANTIGENS AS ARTHRITOGENIC ANTIGENS IN RHEUMATOID ARTHRITIS

There is some evidence supporting the statement that citrullinated peptides are likely arthritogenic autoantigens [3]. In a recent study, citrullinated peptides exacerbated arthritis in a two-hit event. Synomologus macaques carrying the HLA-DRB1SE called H6 haplotype were immunized with a pool of three citrullinated peptides, fibrinogen, vimentin and aggrecan, but there were no clinical manifestations. Then, intra-articular injection of the three peptides and incomplete Freund's adjuvant caused severe and prolonged arthritis, preferably in animals carrying the HLA-DRB1SE, called H6 haplotype, whereas animals injected with incomplete Freund's adjuvant alone exhibited only transient arthritis [52].

CONCLUSION

There is oral and gut dysbiosis in RA. In dysbiosis, peptides from specific components of gut microbiota, such as *P. copri*, as well as self-epitopes (T-cell epitopes) of highly expressed proteins in synovial membrane, sharing sequence homology, are targets of T cells, activate intestinal Peyer's patch Tfh cells which then migrate to distal lymphoid tissues and lead to the formation of germinal centers producing autoabs. These antigen-specific Tfh cells help germinal center B cells to augment autoAb production to microbial and self-protein. One expected requirement for this series of events is a high affinity binding of peptide/HLA/T cell receptor.

These findings have therapeutic implications. An obvious intervention is to keep oral hygiene and treat periodontitis. The rationality of this approach is to stop feeding autoantigens. The use of probiotics to restore gut microbiota may be another approach. Although few small studies showed little benefit [53], this approach requires further investigation. For instance, SCFAs treatment of mice ameliorated CIA and this was associated with the reduction of Th1 cells and increase in Tregs, but exacerbated antibody-induced arthritis [54]. In a different strategy, it may not be far away the time where bifunctional nanoparticles coupled to citrullinated autoantigen epitope and lytic complement-activating peptide are therapeutically used in RA patients to deplete autoantigen epitope-specific B cells [55].

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Conflicts of interest

There are no conflicts of interest.

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- ■■ of outstanding interest

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