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Rheumatoid Arthritis and the Mucosal Origins Hypothesis: External Protection Becomes Internal Destruction

V. Michael Holers[1], **M. Kristen Demoruelle**[1], **Kristine A. Kuhn**[1], **Jane H. Buckner**[2], **William H. Robinson**[3], **Yuko Okamoto**[1], **Jill M. Norris**[4], **Kevin D. Deane**[1]

[1]University of Colorado Denver, Division of Rheumatology, Aurora, CO, USA

[2]Benaroya Research Institute, Seattle, WA, USA

[3]Stanford University, Division of Immunology and Rheumatology, Stanford, CA, USA

[4]Colorado School of Public Health, Department of Epidemiology, Aurora, CO, USA

Abstract

Individuals at high risk for the future development of seropositive rheumatoid arthritis (RA) can be readily identified for translational research and disease prevention studies through the presence of highly informative and predictive patterns of RA-related autoantibodies, especially anti-citrullinated protein antibodies (ACPA), in the serum. Recent findings in serologically positive individuals without arthritis, designated ACPA+ At-Risk, have demonstrated the presence of mucosal inflammatory processes associated with the presence of local ACPA production. In other At-Risk populations, local RA-related autoantibody production is present even in the absence of serum autoantibodies. Additionally, a proportion of At-Risk individuals exhibit in the lung local mucosal ACPA production, radiographic small airways disease and sputum hyper-cellularity as well as increased neutrophil extracellular trap (NET) formation. Other mucosal sites in At-Risk individuals also exhibit autoantibody production, inflammation and/or evidence of dysbiosis. As the proportion of individuals who exhibit such localized inflammation-associated ACPA production is substantially higher than the likelihood of an individual developing future RA, this raises the hypothesis that these antibodies are generated in the mucosa to play biologically relevant protective roles. Identifying the mechanisms that drive both the generation and loss of externally focused mucosal ACPA production and promote systemic autoantibody expression and ultimately arthritis development should provide insights into new therapeutic approaches to prevent RA.

Introduction

It is now known that seropositive rheumatoid arthritis (RA), which occurs in individuals who have both genetic and familial risk factors for this disease[1–4], begins as a prolonged multi-

Correspondence to V.M.H. at michael.holers@ucdenver.edu.

Author contributions

V.M.H. wrote the first draft of the manuscript, and all other authors researched data for the article and contributed substantially to discussions of its content. All authors reviewed and revised the document prior to submission. The authors also appreciate the early input by Professor Ranjeny Thomas to the conception of this review.

Competing interests

The authors declare no competing financial interests.

year period of serologically-detectable RA-related autoimmunity that is associated with systemic inflammatory biomarkers but the absence of clinical or histologically defined inflammatory arthritis[5–9]. Serum autoantibodies present at this time include multiple isotypes of both anti-citrullinated peptide/protein antibodies (ACPA) and Fc domain-recognizing rheumatoid factors (RF) in patterns highly predictive of the future onset of joint disease[5,6,9,10]. This period can be defined retrospectively as the “pre-clinical” period of RA in subjects who eventually develop the disease[11] and an At- Risk status in the population when studied in a cross-sectional or longitudinal manner prior to arthritis. This condition is then followed by the development of early clinically detectable inflammatory arthritis (IA) and/or classified RA, the latter of which itself proceeds as a chronic arthritis and disease process that can also involve the lung and other target organs.

Epidemiologic and translational research studies of patients with early (< 1 year from diagnosis) or longer standing active RA suggest that mucosal exposures and/or dysbiosis may have played causal roles during the At-Risk state in the development of RA[2,12–16]. However, despite the support for a “mucosal origins hypothesis”, ie that RA development begins at mucosal sites and then transitions to involve the synovial joints, these study designs are limited in that they do not allow one to identify the specific mucosal processes present at the prior time point(s) during which such exposures would have influenced the concurrent autoimmune phenotypes as well as subsequent disease evolution and arthritis development. In this regard, it is also often assumed that a key factor in early RA development is the loss of self-tolerance to citrullinated self-antigens. However, studies of At-Risk as well as control populations strongly suggest that mucosal, and likely systemic, IgA isotype ACPA and RF generation is a rather common finding and is associated with local mucosal inflammation[17,18]. These findings suggest that the likely most important early event in the pre-clinical development of RA is not a loss of tolerance to self-antigens but rather the loss of the mucosal barrier function and systemic spread of an IgG ACPA response. With those issues in mind, and to address the mucosal origins hypothesis in the context of findings in subjects At-Risk for future RA, this manuscript will review the available evidence linking the presence of RA-related autoimmunity in specific At-Risk populations with the concurrent presence of local mucosal inflammation and autoantibody production, mucosal dysbiosis and/or systemic immune dysregulation consistent with mucosal inflammation.

Development of seropositive RA

RA is an autoimmune disease that evolves over decades and is manifest as both a locally joint destructive and systemic illness[4,19]. The disease is characterized by widespread inflammation as well as local joint-based signs and symptoms ranging from arthralgias, a term which has recently been specifically defined for research purposes to encompass seven factors (symptom duration <1 year, symptoms of metacarpophalangeal (MCP) joints, morning stiffness duration ≥60 minutes, most severe symptoms in early morning, first-degree relative (FDR) with RA, difficulty with making a fist, and a positive squeeze test of MCP joints)[20], to swelling, pain and inflammatory synovitis that are clinically definable through techniques such as physical examination, imaging and synovial biopsy [reviewed in[19,21]]. Osteopenia and local erosions commonly occur in RA, likely through inflammatory

and remodeling processes that alter relative rates of bone formation and degradation by osteoblasts and osteoclasts, respectively[22].

RA exhibits a prevalence of 0.5–0.8% in the general population, and an ~3–5-fold increase in FDRs [reviewed in[23]] which appears to be due to both genetic and environmental influences[24–26]. Patients receive a diagnosis of RA based on the 1987 revised ACR or 2010 ACR/EULAR classification criteria[27,28]. In some patients RA first appears clinically as an undifferentiated inflammatory arthritis (IA)[29,30].

The term 'RA' encompasses two major subsets of disease, seropositive and seronegative, that exhibit overlapping but individually distinct pathogenic mechanisms and clinical courses [reviewed in[21,26,31]]. Seropositive individuals exhibit substantial overlap of expression of two primary autoantibody systems: 1) ACPAs, with this posttranslational modification found in an antigenic form on fibrinogen, vimentin, type II collagen, histones and enolase[32,33], and 2) antibodies to the Fc domains of self Ig molecules, designated rheumatoid factors (RF)[34]. Additional autoantibodies to modified protein antigens (AMPA) [reviewed in [35]], such as anti-carbamylated protein antibodies[36] and antibodies to malondialdehyde-acetaldehyde modified epitopes[37], have been identified in patients with RA, although the specificity for RA of AMPA and at what point in the pre-clinical to clinically apparent disease transitions AMPA develop are less well defined features[38,39].

RA can be driven by a variety of genetic and environmental factors that lead to a heterogeneous disease process with variable autoantibody positivity. In terms of genetic factors, there are many genes which associate with RA, including high risk HLA-DR alleles containing the shared epitope (SE)[40] and *PTPN22*[41], as well as many other genes with low relative risk variants[42]. Systemic targeting of citrullinated proteins is likely facilitated by the preferential interactions of peptides bearing citrulline with SE-containing DR molecules[43–45]. Heterogeneity in RA disease phenotypes is also likely to be influenced by environmental factors[25,46]. In addition, there are epigenetic changes that are associated with RA and affect lymphocytes, fibroblast like synoviocytes (FLS) and other cell populations[3,4,47,48]. These epigenetic changes represent key means by which environmental factors could influence gene expression and disease heterogeneity.

In subjects who eventually develop seropositive RA, there is a prolonged asymptomatic pre-clinical period without evidence of joint involvement that is characterized by the presence of serologically detected ACPA, measured through clinical tests designated anti-CCP (cyclic citrullinated peptide) antibodies, and RF in a process that typically occurs for 3–5 years prior to the onset of clinically apparent and classified disease [reviewed in[49]] (FIG. 1). Shortly before the onset of clinically apparent arthritis, systemic levels of cytokines/chemokines increase in a very heterogenous manner, both with regard to the number and the pattern of individual mediators that are elevated in an individual subject[10]. An increase in epitope spreading also occurs which stably encompasses a wider variety of citrullinated targets[9,50], and avidity maturation of autoantibodies is present[51].

In addition, effector functions of ACPA evolve during the development of RA[51,52]. For example, it has been shown that altered ACPA Fc domain galactosylation and

fucosylation[53], as well as excessive Fab glycosylation[54], is a common finding in patients with RA and those who are transitioning from the arthralgia state to IA and classified RA. Notably, recent studies have suggested that IL-23 and Th17 cells regulate the β-galactoside α2,6-sialyltransferase 1 activity in B cells that controls the extent of glycosylation of Ig molecules and their pathogenic potential, and that changes in these cytokines drive the disease-enhancing changes in secreted autoantibodies during the clinical transition to arthritis[55]. Importantly, autoantibodies have also been identified in patients with RA that demonstrate effector functions related to osteoclastogenesis[56] and joint pain secondary to chemokine release[57], although the point during disease development during which these appear is less certain. Perhaps most intriguingly relative to mucosal studies, it has been found that IgA ACPA and RF isotypes long precede the development of classified RA[58], although the relative timing of appearance of each serum RA-related autoantibody isotype is not yet clearly defined. In addition, IgA RF contains in patients with RA a high proportion of mucosally-related secretory chain[59], although the time point of appearance of this feature is again uncertain.

Serologic immune alterations during disease evolution

Studies in the pre-clinical time period have primarily relied on two experimental approaches. The first has been to utilize annotated sera that have been collected previously for other purposes, the origin of which includes blood banks and population studies[5,6,8,60] as well as the United States Department of Defense Serum Repository[7]. As summarized above, despite the inherent limitations of subjects not being studied and classified with regard to arthritis status at the same time as the sample acquisition, many insights have been generated from these approaches. However, a more recent approach has been to perform studies of individuals with an ACPA+ At-Risk status, which is a method that has increasingly helped to define how the earliest immunologic abnormalities develop and what may be the early “drivers” of disease. These individuals are recruited through many different means, including recruitment of higher risk first-degree relatives (FDRs)[61,62], outreach at community health fairs or other events to identify serum ACPA+ At-Risk subjects[63] and the creation of referral networks to specifically identify subjects in primary care with arthralgias suggestive of pre-clinical RA who are subsequently followed for IA and classified RA evolution[64]. Ongoing population recruitment efforts include screening of biobanks and electronic health records, social media outreach, and the increasing referrals to rheumatologists of patients without IA or RA who are found to be ACPA+.

This heterogeneity in sources of samples may lead to some differences in the findings from retrospective studies. Nevertheless, a number of important observations have been made using these approaches (BOX 1). Key findings in serum samples from studies of At-Risk populations include the presence of elevated chemokines/cytokines/inflammatory biomarkers[62,65,66] and altered T cell phenotypes[67,68] as well as expanded autoantibodies[61,65,69–71]. Importantly, many of these biomarker patterns mirror those found in the serum from retrospectively studied populations and strongly support that these individuals are indeed in a pre- clinical period of RA development. Results of additional phenotypic studies are described below.

Stage-specific environmental factors

The potential roles of environmental exposures in the development of RA have been extensively reviewed[23,25]. With regard to the At-Risk population, though, very few long-term prospective studies have been performed whose goal would be to understand how stage-specific exposures influence the initial pre-clinical development of RA-related autoimmunity and subsequent clinical and/or immune transitions. Nevertheless, a number of informative cross-sectional and short-term longitudinal studies have been performed in these subjects (TABLE 1).

A well-accepted association in RA is the relationship between heavy smoking and the risk of development of seropositive RA, with the predominant risk being in subjects with SE[26]. The exact stage- specific timing of smoking effects during the pre-clinical to classified arthritis natural history of RA is not well understood, but several studies have provided insights. For example, initial studies of At-Risk subjects in a well studied cohort evaluated in real time have revealed that heavy smoking is associated with asymptomatic RF positivity[72]. In a Swedish twin study, heavy smoking impared a higher risk of being ACPA+ without RA, and without a relationship to SE in the preclinical period[73]. In addition, in a large (>40,000 subjects) population study in the Netherlands, an ACPA+ status was associated with smoking as well as older age and joint symptoms[74]. Further support for a role of smoking exposure in the future development of RA was provided by a Swedish population study where smoking was associated with an increased risk of development of both seropositive and seronegative RA[75]. Similarly, in a Japanese non-RA cohort, a history of smoking was associated with a positive RA-related autoantibody status, but interesting only when both ACPA and RF were present[76]. In a further analysis of the same population, ACPA and RF were shown to associate with each other, and smoking was associated with a higher level of ACPA, but the presence of SE was not associated with an ACPA+ status, and no interaction was found between SE, smoking and autoantibody positivity in the non-RA population[77]. Thus, it appears that smoking plays an important role in the initial development of ACPA, but without a clear relationship to SE status, which itself appears to play a more important role in the transition to classified RA.

With regard to other factors, a history of birth control pill use is inversely associated with the presence of RF positivity[72]. These results are consistent with findings reported when studying patients with classified RA and suggest that these environmental exposures can play key early roles in pre-clinical RA.

Conversely, particulate air pollution was not found to be associated with RA-related autoimmunity in At-Risk subjects[78] in the same manner as patients with classified RA[79], suggesting that this exposure may play a later role in the development of RA, perhaps promoting the transition from RA-related autoantibodies alone to the initial synovitis. Additional results from the Nurses Health Studies have suggested that an increased body mass index (BMI) in conjunction with expanded ACPA repertoire is associated with elevated future risk of developing RA and accelerated transition to arthritis[80]. Studies from the Epidemiologic Investigation of RA (EIRA) have also suggested that exposure to silica increases the risk of future development of RA[81]. Other occupational exposures, hormonal factors, dietary intake and alcohol use have demonstrated variable results, and the roles of

these exposures in the pre-clinical stages of diseases are less well understood [reviewed in[25]].

An intriguing finding has been an inverse relationship between the presence of RA-related autoantibodies in At-Risk populations and intake of omega-3 fatty acids as well as lipid biomarkers that measure levels of these key anti-inflammatory molecules. Because of the potential relationship of this exposure to the chronic local inflammation that underlies the mucosal origins hypothesis, these findings are reviewed in more depth. Notably, there is both established and emerging evidence that poly-unsaturated fatty acids (PUFAs) derived from the omega-3 FA biosynthesis pathway may play an important role in RA treatment and prevention. Omega-3 PUFA supplementation in patients with classified RA has been shown to reduce signs and symptoms of inflammation and lead to decreased use of other therapeutic agents[82,83]. Population-based approaches have demonstrated that increased intake of fish rich in omega-3 PUFAs or direct supplementation with PUFAs are associated with a reduced risk for developing RA[84]. With regard to At-Risk subjects, it has recently been shown that increased omega-3 FA intake as well as higher levels of the red blood cell (RBC) omega-3 FA pathway eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are associated with a decreased risk of RA-related autoantibody positivity[85]. Specifically, in those studies the likelihood of anti-CCP2 positivity in subjects was inversely associated with total omega-3 FA% in RBCs (odds ratio: 0.47; 95% CI 0.24, 0.92, for a one standard deviation increase), and anti-CCP2 positive cases were less likely than controls to report omega-3 FA supplement use (odds ratio: 0.14; 95% CI 0.03, 0.68). Intriguingly, the strongest effect was found in SE+ individuals[86]. In further observational studies of ACPA+ subjects without synovitis at baseline, lower levels of RBC omega-3 FAs were associated with increased risk for progression to IA[87].

Mucosal origins hypothesis

The mucosal origins hypothesis suggests that the disease process in subjects who eventually develop RA originates at one or more sites in the mucosa. The theory historically derived from an early suggestion that RA was an infectious illness, a conclusion that was subsequently been shown to be incorrect. Later suggestions included the possibility that RA developed through a cross-reactivity to bacterial, viral and/or mycobacterial antigens carried in such organisms as Proteus [reviewed in[88]]. More recently, the finding that *Porphyromonas gingivalis,* a major cause of periodontitis, encodes an enzyme capable of citrullinating proteins, including enolase, and cross-reactivity of these targets with patient ACPA suggested another means by which mucosal dysbiosis could drive or influence disease development[89]. Nevertheless, the hypothesis remains unproven, although an increasing number of studies are consistent with a major role for the microbiome in RA development.

The microbiome and autoimmunity

Increasing evidence from both murine and human translational research studies points to the important role of the microbiome that is present in the intestine and other mucosal as well as epithelial sites in modulating the immune, as well as autoimmune, response [reviewed in[90]]. Food intake and other environmental exposures play major roles in the development and

maintenance of microbial kingdoms, including bacterial, fungal, helminth and viral organisms. Major effects on the immune system are played by mucosal exposure to specific microbial antigens as well as metabolic products of biosynthesis and energy generation/utilization[2,91].

There are many challenges to the study of the microbiome in humans, including lack of direct access to most mucosal sites, difficulty in isolating and expanding many species in a manner that replicates the local mucosal ecosystem, an incomplete understanding of microbial genotype:phenotype relationships, substantial changes introduced to the microbiome by unrealized food exposures such as dietary emulsifiers and non-caloric sweeteners, long-term imprinting by the fetal microbiome whose effects cannot be discerned years later, cultural differences that affect microbial communities, and the rapid changes that can ensue following alterations in the environment which are perhaps best demonstrated by the effects of changing food intake on the microbial community in the gastrointestinal tract[90]. Nevertheless, studies of murine models of disease and an emerging number of human translational research studies have pointed to a causal or modulating role for the microbiome in human autoimmune disease.

Cross-talk with the local immune system—Although they may vary from one organ to another, there are a number of means by which the local mucosal immune system can interact with, respond to and constrain the microbiome (FIG. 2). These include induction and inhibition mechanisms that are strongly influenced by cells of the adaptive immune system as well as distinct types of mononuclear phagocytes[90]. An important feature of the cross-talk process between the immune system and the microbiome is the mucosal barrier, which is created not only by inter-epithelial cell tight junctions but also by the integrity of the mucous layer and serves as a component of the “mucosal firewall” [reviewed in [92]]. Additional components of the firewall include anti-microbial peptides, encompassing in part the microbicidal protein Reglly and related families[93], antibodies, macrophages, dendritic cells, Treg and Th17 T cells. Also important in mucosal defense from infection are neutrophils, which are attracted to the lumen through polarized secretion by epithelial cells of IL-8 as well as bioactive lipids, especially hepoxilin A3 [reviewed in[94]].

CD4 and CD8 T lymphocytes also continuously surveil antigens in this region, responses to which are regulated by cytokines including IL-17, IL-10, IL-22, IL-23, IL-6 and TNF family members, in addition to other populations such as Treg cells. In addition, specialized T lymphocytes defined as MAIT (mucosal associated invariant T) cells, whose innate immune functions and activation state are intimately tied to MR1 ligands that are elaborated during mucosal infection and inflammation, are present and produce IL-17 and Th1 cytokines[95]. Type 3 innate lymphoid cells (ILC3) contribute to the mucosal immune response through production of IL-22, a cytokine that stimulates production of RegIII lectins in response to dysbiosis[96] as well as controls epithelial cell fucosylation and through that mechanism provides a carbohydrate source utilized by certain microbial subsets[97]. Also present are mucosal homing receptor[98] $\alpha_4\beta_7^{high}$ $CD4^+$ T cells that are $CCR5^{high}$ and $CXCR4^{low}$ and which undergo antigen-specific activation in the development of mucosal inflammation[99].

With regard to antibodies, IgA is the major Ig isoform produced at most mucosal surfaces, and while the circulating blood levels are low, IgA is the most highly produced Ig isoform in the body[100]. Importantly, both T-dependent and T-independent means regulate the production of this mucosal isoform[101]. IgA exists as monomeric and polymeric forms of IgA1 and IgA2 sub-isotypes as well as polymeric IgA linked by the J chain. The IgA receptor (IgAR1) is found on neutrophils, monocytes, eosinophils and a subset of dendritic cells, and IgA functions to enhance the respiratory burst and inhibit bacterial adhesion.

There are several contrasting roles ascribed to this isotype. Its primary role is to recognize and neutralize pathogens and exotoxins at the mucosal surface, and in that way to regulate the microbiome, maintain homeostasis and protect intestinal and other barriers from pathogens. A key mechanism is to block infection and invasion of the mucosa through exclusion from transport across the epithelium and clearance by peristalsis in the gut[102]. IgA plasmablasts are primarily generated in Peyer's Patches and can circulate to secrete Ab at other mucosal sites. While IgA is view primarily in the context of mucosal immunity, when present systemically at high levels in immune complexes, it can damage target tissues, including the joint[103].

In another sense, secreted IgA antibodies can be considered to be non-inflammatory as they are typically not strongly complement activating and are resistant themselves to proteolytic degradation[104]. Thus, the pro- versus anti-inflammatory roles of IgA must be considered to be context-dependent, and likely co-existing. Beyond IgA, there are prominent roles for IgM antibodies as well as IgG antibodies and their bidirectional transport by the neonatal Fc receptor FcRn in the regulation of the microbiome and control of local pathogens, perhaps most prominently in the cervicovaginal fluid [reviewed in[105]]. Notably, bacteria which penetrate the mucosal "demilitarized zone" are recognized by locally produced IgA isotype antibodies that are typically polyreactive[106], and if they avoid neutralization and elimination can more readily cross the epithelial layer and have antigens be presented by dendritic cells[107].

In addition to bacteria, viruses and other species can profoundly influence the mucosal immune response[90]. Examples include both bacteriophage as well as DNA and RNA viruses[108]. Other organisms including mycobacteria, fungi and helminths can play key roles in regulating local and systemic immune responses[90]. To date, little information is known regarding the potential influence of non-bacterial microbiome on the pre-clinical initiation or evolution of autoimmune diseases.

Another important feature of the mucosal immune response is the regulatory effect of the microbiome on local cells, as demonstrated by the generation of bacterial metabolites that modulate differentiation and effector functions. For example, the microbe-derived short chain fatty acids butyrate, propionate and acetate all strongly affect the differentiation of mucosal Treg cells[109].

Evidence of disease modulation from experimental models—Several murine models of arthritis have been studied in order to define what changes occur to the mucosal microbiome during the evolution of experimental arthritis and conversely how those can

affect the development of systemic autoimmunity and synovitis. These studies have in aggregate strongly supported a role for microbiome influences on arthritis development through several mechanisms.

In the type 2 collagen (CII)-induced arthritis (CIA) model, previous studies have defined important pathogenic roles for the ACPA response and the potential beneficial clinical and autoimmune outcomes following modulation of that response using toleragenic approaches to decrease the level of pathogenic ACPA[110]. More recent studies have demonstrated dysbiosis when evaluated at the terminal point of study in the CIA model and that transfer of fecal material from these arthritic as compared to non-arthritic mice accelerates the development of arthritis in recipient germ free mice[111]. Additionally, increases in IL-17 and splenic CD8 and Th17 lymphocytes were seen with this treatment, in contrast to decreases in dendritic, B and Treg cells. Other studies have demonstrated that, while modulation of the microbiota through the use of broad spectrum antibiotics prior to the induction of CIA resulted in a modest reduction in disease severity, antibiotic use during the late phase of CIA resulted in nearly complete suppression of arthritis[112]. The later effect was in part due to a significant impairment in the ability of anti-CII antibodies to activate complement ex vivo, while the earlier depletion was associated with decreased circulating inflammatory cytokines and anti-CII antibodies along with delayed IL-17A and IL-22 production in the intestine. Perhaps most notably, these data establish that the microbiome can exert profoundly different effects at time points during the pre-clinical to clinical evolution of arthritis, a finding which is likely quite germane to the study of the potential roles of the human microbiome during initiation of autoimmunity and through the multiple stages of the natural history of RA. In addition, this result suggests that all of the effects of the mucosal immune cell interactions with the microbiome may not be manifest only locally, as subsets of lymphocytes in the mucosa are also known to re-circulate and populate other sites, and in that process influence the immune and autoimmune response in other tissues[113]. Indeed, it may be that “licensing” of T and B cells to become pathogenic must occur through either local activation or passage of these cells through mucosal sites, especially the gut but perhaps also the lung, following which they can then elaborate their pathogenic potential in target tissues during autoimmune disease processes.

Other models of RA have demonstrated features that may also be relevant to the roles of microbiota in human disease. For example, the K/BxN TCR transgenic mouse is a model in which CD4 T cell driven autoantibody recognition of the ubiquitous autoantigen glucose-6-phosphate isomerase (GPI) results in an immune complex-driven arthritis[114]. In a germ free environment, transgenic mice exhibit a delayed onset of arthritis and reduced severity, while segmented filamentous bacteria (SFB) are capable to modulate the development of autoantibodies to GPI by activating and recruiting of Th17 and Tfh cells within the intestine[115,116]. In addition, studies of the human DR4/DQ8 humanized mice have demonstrated that treatment with a human commensal organism, *Prevotella histicola,* suppressed the development of CIA[117]. Mechanistically, this treatment altered CD103 dendritic cells, myeloid suppressors and Treg cells in the intestinal mucosa, resulting in suppression of antigen-specific Th17 responses as well as increases in antimicrobial peptides and tight junction proteins.

Inflammation in At-Risk populations

There are several systemic immune abnormalities found in retrospective serum studies as well as present in ACPA+ and other At-Risk populations that suggest the presence of ongoing mucosal inflammation (FIG. 3). One is the predominance of IgA isotype ACPA and RF in retrospectively studied preclinical samples[58] as well as the presence of serum IgA ACPA and RF in At-Risk populations[18]. More compelling, however, are data obtained from an analysis of plasmablasts from At-Risk populations. These are relevant cells to study as during an immune response, peripheral blood plasmablasts arise from both naïve and memory B cells that are activated by inciting antigens, including those at mucosal sites. To evaluate these cells, plasmablasts derived from RA-related autoantibody positive At-Risk subjects as well as matched controls and individuals with early RA were sequenced and characterized utilizing a barcode-enabled single cell antibody methodology[118]. Notably, while total plasmablast levels were similar in RA-related autoantibody positive At-Risk individuals and controls, a markedly increased frequency of IgA+ as compared to IgG+ plasmablasts was observed in the At- Risk individuals as compared to the other groups, including patients with early RA[119]. Consistent with these findings, At-Risk individuals also demonstrated serum IgA anti-CCP and IgA ACPA peptide-specific antibodies[119].

Beyond this, plasmablasts from a sub-group of At-Risk subjects underwent paired heavy and light chain repertoire sequencing. Phylogenetic trees of paired IgG and IgA sequences were generated[118–120]. From these data, clonal families of antibodies were identified that use the same heavy-chain V and J gene segments, as well as the same light-chain V and J gene segments, consistent with their inter-relatedness during development. Combined IgG + IgA phylogenetic trees were also generated to demonstrate the relationship between sequences across these isotypes, and remarkably a substantial number of dual IgG/IgA cross-isotype clonal families of varying sizes were observed[119]. These data are consistent with an ongoing immune response that has a pronounced mucosal origin and/or contribution.

Other studies of At-Risk individuals have also demonstrated findings consistent with a state of chronic immune stimulation and inflammation[121], which in the absence of another tissue or organ site of origin is a finding very consistent with mucosal inflammation. For example, studies of peripheral blood cells exhibited unique phenotypes in CD4+ peripheral T cells consisting of elevated citrullinated histone H3 (cit-H3) as well as increased stimulated production of IL-2 and Th17 cytokines but fewer Th2 cytokines[121]. With regard to the underlying immune mechanisms of these particular findings, the abnormal features appear to be due to the impaired induction of PTPN22 in T cells. PTPN22 is a phosphatase that also suppresses citrullination independently of its phosphatase activity and when expressed as an RA-associated variant in neutrophils promotes accelerated NETosis[122].

In addition, in the same study of At-Risk individuals attenuated phosphatase activity of PTPN22 was found to result in aberrant expression of IL-2, ataxia telangiectasia mutated (ATM), and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), which is associated with a hyper-proliferative phenotype[121]. Furthermore, diminished non-phosphatase activity of PTPN22 led to hyper-citrullination mediated by PADs and reduction of expression of Th2 cytokines. Notably, these T cell phenotypic changes identified in At-Risk subjects closely mirror prior results obtained in patients with longstanding RA, studies

which showed that patients with active arthritis also exhibit pronounced changes in the metabolic activity of CD4+ T cells where they do not metabolize glucose as efficiently as controls, resulting in less intracellular ATP and enhanced sensitivity to apoptosis[123,124]. Mechanistically, the defect was related to a lower induction of PFKFB3, with resultant metabolic shifts by hypoglycolytic T cells through diversion of glucose toward the pentose phosphate pathway, more NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) and utilization of intracellular reactive oxygen species (ROS)[123]. In aggregate, these changes left the T cells energy deprived, apoptosis sensitive and autophagy-resistant. Subsequent studies demonstrated that insufficient activation of ATM drove T cells towards a hyper-inflammatory Th17 and Th1 lineage, bypassing of the G2M checkpoint and hyper-proliferation[124]. Notably, correction of the ROS changes reversed these defects both in vitro and in an in vivo model. Finally, one additional finding in support of a chronic infection, likely viral in nature, being relevant at an early this point in disease is the finding of an elevated type I interferon score in individuals who have transitioned from a late pre-clinical phase to classified RA[64].

In sum, several lines of evidence from peripheral blood and serum studies have strongly suggested the presence of an ongoing inflammation, likely mucosal in origin, and an immune response that is associated with a state of heightened immune reactivity and inflammation. As the At-Risk individuals do not manifest evidence of arthritis, as assessed by MRI, physical exam and even synovial biopsy, the presence of an asymptomatic chronic mucosal process associated with these peripheral blood changes is very feasible.

Tissue-specific mucosal Inflammation

Also relevant in support of the mucosal origins hypothesis in RA development are studies in At-Risk populations demonstrating the concurrent presence of inflammation and RA-related autoantibody production in informative mucosal sites. To that end, this section will review available evidence in tissue sites when assessing both the presence of informative site-specific changes in early RA as well as what is known in the same organ when one is studying At-Risk populations.

Intestinal Biomarkers—With regard to RA, seminal studies performed in patients with new-onset rheumatoid arthritis (NORA) demonstrated a relative expansion of fecal *Prevotella copri* in a substantial number of patients[13]. Although no microbiome-based replication studies have been published, findings in a separate study population have demonstrated an enhanced IgG and IgA humoral and Th1 T cell immune response to this organism in a subset of patients with established RA[125]. Another study of patients with established RA identified alterations in fecal *Lactobacilli*[15], changes which partially respond to drug treatment, and in a separate cohort study an expansion of rare species with the potential to modify experimental RA[16] was found in patients. Notably, it is unknown as to whether the changes noted in these sites in patients with active disease are causal or secondary to inflammation or drug therapy, and why the microbiome findings differ from one research group to another. No comprehensive studies of the fecal or intestinal microbiome have been reported in RA At-Risk populations, but such studies are underway and have suggested the presence of local ACPA production and dysbiosis.

Lung Biomarkers—In addition to the risk factors described above that have been largely identified in epidemiologic studies of subjects with RA, and the demonstration that ACPA and RF are produced in inducible bronchial-associated lymphatic tissue (iBALT) in patients with chronic RA[126], there have been compelling studies of early RA that have supported the presence of substantial pulmonary inflammation in conjunction with arthritis. These findings have included the presence of histologic structural abnormalities as well as inflammation and ACPA production of a similar nature as in the joint in lung biopsies and BAL fluid from early RA cases[127,128]. Microbiome studies of the BAL of similar early RA patients demonstrated that the microbiota was significantly less diverse and abundant when compared to matched healthy controls studied in parallel, and that the changes were quite similar to patients with sarcoidosis[12]. The dysbiosis could be attributed to the reduced presence of several genus and taxa. In addition, several clades correlated with the levels of autoantibodies both in the BAL and systemically, and the genus *Pseudonocardia* was the only taxa over-represented. Whether these same dysbiotic changes are seen in At-Risk subjects has not been reported.

To assess the presence of lung abnormalities and local RA-related autoantibody production in At-Risk populations, though, two techniques have primarily been used. The first was high resolution computerized tomography (HRCT), which demonstrated the presence in ~2/3 of At-Risk subjects of small airways abnormalities in the absence of arthritis that were consistent with airway inflammation, with the joints being assessed and negative for inflammatory arthritis by both clinical examination and in a subset of subjects by MRI[129]. Notably, the presence by lung HRCT of small airways abnormalities (bronchial wall thickening, bronchiectasis, bronchiolitis and air trapping) was not dependent upon a history of current or prior smoking, which suggested that other etiologic factors must be involved. In addition, subjects were not aware of any pulmonary problems and did not demonstrate evidence of abnormalities by pulmonary function testing.

The second technique utilized in At-Risk populations has been induced sputum collection, a validated methodology that importantly assesses changes in the same small airways as identified by HRCT[18,130]. This technique has demonstrated the presence of IgA and IgG RA-related autoantibodies in the lung of At-Risk subjects even in the absence of serum ACPA or RF[18]. Analysis of the ratios of specific to non-specific IgA and IgG demonstrated that the RA-related autoantibodies were produced locally. Importantly, local IgA anti-CCP generation was also closely associated with the concurrent presence of elevated inflammatory cells (neutrophils and macrophages) and increased endogenous NET formation in the sputum, as assessed by the detection and quantitation of NET remnants[130]. In aggregate, ~25% of FDRs or individuals with serum autoantibody positivity appeared to demonstrate the presence of local RA-related autoantibody production. Also, a subset of subjects demonstrated systemic ACPA but no pulmonary inflammation, suggesting that a separate mucosal site might be involved in those individuals in the generation of systemic ACPA.

In addition, sputum studies comparing individual ACPA reactivities identified an increased number of sputum ACPA peptide reactivities in RA subjects compared to At-Risk subjects[131]. While cross-sectional, this finding suggests that epitope spreading, which is well

described in the blood during the preclinical period leading to RA onset, may also occur at mucosal sites. Furthermore, in serum anti-CCP positive subjects with lung disease but no joint disease, lymphoid aggregates consistent with iBALT were highly prevalent, further supporting RA-related immune reactivity in the lung in At-Risk individuals[132].

Periodontal Biomarkers—Periodontal inflammation and chronic periodontitis, *Porphyormonas (P.) gingivalis* infection and a relationship to citrullinated enolase reactivity have long been associated with RA and RA severity [reviewed in[133]]. Although the relationship between RA and periodontitis and has been challenged through population-based studies, such as those within the EIRA cohort[134], there remain substantial data supporting a close relationship between the ACPA response, the specific *P. gingivalis* peptidyl arginine deiminase, and RA risk.

Other factors have also been implicated in linking disease to periodontitis and ACPA production, including an elevated abundance of *Anaeroglobus geminatus* in periodontal fluid in patients with NORA that was correlated with the presence of ACPA and RF, and an increase in Prevotella and Leptotrichia species in patients with NORA irrespective of the presence of periodontitis [14]. In other studies, *Aggregatibacter actinomycetemcomitans* has been associated with periodontitis in vivo and the capacity for hyper-citrullination ex vivo[135].

As opposed to lung studies, there is a more limited understanding of the relationship between the RA At-Risk state and periodontitis, as studies of that population are just being completed. Prior studies have demonstrated an increased prevalence of antibodies to *P. gingivalis* in At-Risk populations[136]. In addition, initial metagenome sequencing reports from the periodontum of At-Risk subjects have begun to emerge, although no conclusions have been made available[137]. Studies of subjects with periodontitis without RA have demonstrated the presence of citrullinated proteins as well peptidyl arginine deiminase enzymes 2 and 4, with both correlated to the level of inflammation, and as well ACPA in a subset of periodontal fluids from patients[138]. Nevertheless, no comprehensive studies of At-Risk populations have been reported that can address the longitudinal relationships between local dysbiosis in the periodontum and the future development of RA. Thus, it remains uncertain as to whether, and in which direction, a causal relationship might exist in the At-Risk and established RA populations.

Other Mucosal Site Biomarkers: Salivary, Urinary and Cervicovaginal—Comprehensive studies of other mucosal sites have not been reported in At-Risk subjects, but are also underway. Salivary IgA ACPA levels have been previously studied in a small cohort of patients with RA, and it was found that ~20% of individuals expressed this phenotype[139]. Notably, in that setting IgA anti-CCP was found to be inversely associated with several poor patient outcomes, thus suggesting that the non-inflammatory, protective functions of the IgA isotype described above may be the predominant effect of expression at this site. To our knowledge, there are no similar studies of At-Risk populations, although in a small set of samples there were marked differences between ACPA levels in induced sputum and saliva of At- Risk subjects[130], suggesting that the two sites operate independently and consistent with the understanding that sputum ACPA levels reflect lung

and not oral sources. Additional studies demonstrated that IgA RF was likely to be produced locally within the saliva and tears in patients with RA, but no studies of At-Risk populations are available[140].

With regard to urinary biomarkers, it is uncertain as to whether ACPA are present in either patients with RA or At-Risk populations. There have been postulated roles in the development of RA for urinary tract infections, perhaps most notably *Proteus* species [reviewed in[141]], but no studies of At-Risk populations have emerged that directly address this relationship. Opportunities for study of the urinary microbiome do exist, however[142].

Finally, the cervicovaginal site has been previously evaluated with regard to the mucosal immune response and susceptibility to transmission of infections such as HIV[143]. Of interest, similar to findings discussed above demonstrating ACPA in the sputum of subjects At-Risk for RA, antigen-specific antibodies for HIV can be found in cervicovaginal fluid in the absence of serum antibodies consistent with a local-limited protective antibody response[144]. While published studies of cervicovaginal-specific biomarkers and ACPA production are lacking, our group has identified elevated levels of cervicovaginal fluid anti-CCP-IgG in premenopausal women with RA as well as a portion of subjects in the At-Risk state[145]. A portion of healthy controls in that study were also identified who were serum anti-CCP negative but had high levels of cervicovaginal fluid anti-CCP that strongly correlated with signs of local inflammation as measured by cytokine elevations. These findings further support the hypothesis that localized and externally focused ACPA production occurs at mucosal sites in response to inflammation and that identifying the mechanisms leading to a loss of externally focused ACPA production will improve our understanding of the development of systemic ACPA in individuals who develop RA.

One limitation to interpretation of the current studies, especially outside of the lungs, is a lack of understanding on a larger population basis what are the levels of mucosal ACPA, how these change over time, and what additional phenotypes and factors associated with antibody status. This is an important area for future investigation.

Potential disease mechanisms

After validation through a series of initial publications that deep phenotyping of RA At-Risk populations is informative, the study of mucosal sites in At-Risk subjects is only now starting to accelerate. No comprehensive longitudinal mucosal studies of At-Risk populations have been reported. Nevertheless, the conjunction of mechanistic studies in other settings with cross-sectional translational human studies in At-Risk subjects provides a conceptual framework in which to consider and then study this process.

Potential protective role of ACPA production—What is perhaps most striking from initial work is that local mucosal IgA and IgG ACPA and RF production is not an infrequent finding, ranging from 15–25% when evaluating a single isotype or autoantibody specificity in cross-sectional studies of the lung in At-Risk populations to ~40% when considering all reactivities. In addition, local lung mucosal RA-related autoantibody production is not a fixed phenotype, as we have observed in preliminary studies that individuals in the At-Risk state both gain and lose anti-CCP antibodies over time (unpublished observations).

With these observations and the knowledge gained from studies of the protective role of mucosal antibodies, the following hypotheses can be put forward (FIG. 4). First, local ACPA production does not reflect an inherent loss of tolerance to self-antigens but is rather a hard-wired form of natural IgA production, perhaps T cell-independent, that is normally externally directed into the mucosal fluid where it likely binds to citrullinated antigens that are released from the neutrophils undergoing NETosis and macrophages which are present as part of a chronic inflammatory state in a mucosal site. Second, multiple mucosal sites have demonstrated the presence of local ACPA production. This includes the lung and periodontum, and as well in preliminary studies the intestinal and CV sites. These findings suggest that RA does not have to begin in a single mucosal region, but may rather originate in one of many that vary from subject to subject. This finding may underlie the very heterogenous manner in which the autoimmune process develops in the pre-clinical period, with neither specific ACPA peptide reactivities[9] nor individual or combinations of cytokines[10] being either necessary or sufficient for the transition to classified RA. Third, it is likely that a major driver which results in the loss of the mucosal barrier and development of a systemic IgG ACPA response is an unresolved state of inflammation in the mucosa. This process could be driven by dysbiosis of bacteria or other microbiota, the nature of which may be similar from one individual to another, or alternately vary substantially across At-Risk subjects. It is important to note, though, that to further validate this proposed process going forward, a clear understanding of the relationships between systemic and mucosal B cells and the BCR sequences that encode ACPA must be generated through paired tissue and peripheral blood studies.

T cells and molecular mimicry as drivers of autoimmunity—There are certainly other factors that should be considered in the process of expansion of local autoantibody production to systemic spread and ultimately development of arthritis. These include the strong association of RA with SE, which implicates antigen presentation and CD4 T cells in its pathogenesis. Studies have demonstrated that CD4 T cells specific for joint derived citrullinated peptides are present in increased numbers and with distinct phenotypes in RA[44,45]. These CD4 T cells likely play a role in the evolution of RA by both promoting tissue specific inflammation and providing B cell help for the formation of high avidity pathogenic ACPA. Molecular mimicry has been proposed as one source of failed tolerance in RA and provides a framework with which to link the microbiome with genetic risk by identifying CD4 T cells that respond to microbial and self-antigens that share similar motifs.

Several approaches are being used to address the molecular mimicry hypothesis. For example, motif analysis has be utilized to identify alpha-viral peptides that have similar motif to type II collagen, a known autoantigen in RA[146]. A peptide sequence motif (DERAA) contained within HLA DRB1*13, a protective allele in RA, has been shown to mediate thymic tolerance in the context of HLA-DQ, but when this molecule is absent, the peptide sequence can be recognized by CD4 T cells_and is cross-reactive with microbial derived epitopes and the self-antigen vinculin[147]. This finding suggests a mechanism by which an initial failure of thymic tolerance is followed by activation with a microbial antigen that then supports a T and B cell response to citruillated-vinculin[147]. Most recently, proteomic tools have been used to perform epitope discovery through elution of peptides

from HLA Class II molecules found in the synovium of RA patients and mass spectroscopy based characterization. This approach has identified novel autoantigens that are cross reactive with microbial proteins for which T and B cell responses are increased in RA patients[125]. As we increase our understanding of the microbial species that participate in the development of RA and determine which autoantigens are early targets of the immune response, the role of molecular mimicry in the development of RA should be further clarified.

It is of course conceivable that molecular mimicry on top of the hard wired IgA response to citrullinated products of inflammation are both necessary to develop classified RA. To address this question, regardless of whether the local mucosal ACPA immune response is meant to primarily react with citrullinated products of dead cells, the potential for molecular mimicry as a driver of an expanded pathogenic mucosal and then ACPA immune response, as recently demonstrated in type 1 Diabetes[148] and consistent with plasmablast studies in patients with chronic RA that recognize citrullinated targets as well as *P.* gingivalis[149], should be further evaluated. In this regard, it is also notable that in subjects with early RA, IgA ACPA are associated with smoking while IgG ACPA are associated with SE, supporting a possible distinction between the local and systemic processes[150].

To better understand these causal mechanisms, and in a similar fashion as above related to the need to address the question of the relationship between systemic and locally produced ACPA, characterizing mucosal T cells and their autoreactivity is essential to further evaluating the potential role of autoreactive T cells in the initial and subsequent development of ACPA.

Other potential inter-related mechanisms—Other inter-relationships may also exist, the first being those that modify local ACPA generation and the second related to the potential ways by which ACPA originating at different mucosal sites, or through distinct inflammatory processes, might influence the subsequent arthritis phenotypes. For instance, alternate primary drivers of autoimmunity may also play key roles, such as inadequate local T cell immunoregulation influenced by genetic or environmental factors, aberrant expression of adhesion molecules and/or cytokines/chemokines, activation of non-toleragenic antigen presenting cells, or engagement of non-traditional APCs such as fibroblasts. Additional modulators of the process may include metabolomic changes that promote systemic spread of the ACPA response, a physical or physiological break in the mucosal barrier, direct or indirect activation of transiting autoimmune cells through enhancement of antigen presentation or alteration of dendritic cell function to expand a response, and elaboration of innate immune factors that promote activation such as LPS, HMGB, CpG or other danger-recognition molecules. In addition, acute infection that is operating on either a normal mucosal or on top of chronic local ACPA production may play an important role in triggering additional pathogenic changes.

Finally, one could ask how chronic inflammation might relate mechanistically to epidemiologic factors such as smoking or lower levels of omega-3 PUFA. Prior studies have suggested that smoking is associated with increased citrullinated proteins in the lung[151]. With regard to the omega-3 PUFA levels and exposures, recent studies have shown that

molecules derived from this pathway, especially bioactive lipid mediators including resolvins and maresins that are biosynthesized *in vivo* from EPA and DHA, are key factors in resolving or reducing inflammation[152]. The observation that RBC levels of omega-3 fatty acids are lower in those progressing to IA suggest that these factors are either present in insufficient quantities or are consumed as part of the excessive mucosal inflammation that is present, and that these two processes, local inflammation and a lack of appropriate levels of omega3 fatty acids, may be both necessary for continued evolution of disease. These anti-inflammatory compounds decrease inflammation through mechanisms including blocking neutrophil and other cell chemotaxis/recruitment, increasing neutrophil apoptosis, and promoting cellular immune switching to regulatory phenotypes (reviewed in[153]). Furthermore, the actions of agents such as resolvins have been shown to reduce inflammation at mucosal surfaces in the lung as well as elsewhere (e.g. gut)[154,155], and to reduce cell activation and immune-complex mediated inflammation in a variety of diseases including IgA nephropathy[153,156,157]. Importantly, as described above, all of these are pathways may be critical to the initiation and propagation of autoimmunity and the ultimate development of arthritis.

As to the second issue, ie the potential influence of mucosal inflammation at specific sites on subsequent arthritis generation, recently distinct synovial subtypes have been identified in RA, including subsets of patients with high-grade synovial infiltrates, vs. a fibrous synovium, vs. other synovial features[158]. Synovial phenotypes in rheumatoid arthritis correlate with response to biologic therapeutics. It is possible that the specific mucosal trigger and IgA plasmablast response, either in general or from specific mucosal sites, could promote a transition to the high-grade synovial inflammatory subtype of RA and that other mechanisms promote transition of the fibrous synovial subtype. It is also of course striking that what are two distinct autoantibody systems, ACPA and RF, can come together and accelerate and amplify joint damage in an experimental model of RA[159], again pointing to the possibility that separate mucosal processes drive distinct autoantibody phenotypes and then the inter-connectedness of the responses drives arthritis.

Therapeutic implications

Although the exact roles of mucosal inflammation in the development of RA are still uncertain, current findings do provide an impetus to consideration of specific mechanism-based therapeutic approaches. For one, while PUFA supplementation has not yet been formally tested in a clinical trial in pre-clinical RA, other trials in animals and humans across a range of diseases (including classified RA) have demonstrated that supplementation does improve clinical parameters [153,160], strongly suggesting that supplementation can result in biologic changes which may prevent development of clinically apparent RA. Additionally, although targeting IL-17 has not proven consistently beneficial in patients with established RA [reviewed in[161]], findings in At-Risk population described above, along with the known role of IL-17 in promoting mucosal inflammation[162], suggest that therapeutic approaches directed at IL-17, or targeting Th17 cells through IL-12/23, may be particularly effective in pre-clinical RA. It is possible that Th17 cells contribute to the evolution of pre-clinical RA via pathways and mediators other than IL-17, including IL-6, IL-21, IL-22 and TNF-α[163]. Other pathways that play roles in epitope spreading following the breaking of tolerance,

including B7/CD28 and CD40:CD40L, also bear strong consideration. The success of co-stimulatory pathway modulation in cancer immunotherapy highlights the importance of these pathways, and we anticipate that key T cell and other co-stimulatory pathways will be identified in autoimmunity[164]. If so, therapeutically targeting these pathways in pre-clinical RA could provide a powerful approach to abrogate the development of RA. Current clinical trials include methotrexate as well as hydroxychloroquine, the latter of which has been shown to exhibit important features such as inhibiting the transition of palindromic rheumatism to classified RA[165] as well as being approved for monotherapy in RA[166] and demonstrated the ability to block several key steps in antigen presentation. And of course, if a specific microbial species or metabolomic signaling pathway[167] is identified as causal in pre-clinical RA, targeting of these processes would be especially informative.

Future directions

In order to test the hypotheses that are laid out above, a number of studies and experimental approaches must continue to be pursued. It is perhaps most important that longitudinal studies of the microbiome, local mucosal phenotypes at multiple sites and systemic RA-related autoantibody production should be pursued in order to determine whether potential causal relationships exist that could drive relevant phenotypic changes in the pre-clinical to clinical transitions. In a related fashion, understanding of the causes of local mucosal inflammation and local ACPA generation should be a research goal. Additionally, exploring the relationships between local and systemic IgA and IgG ACPA production at the molecular and Ig sequence level over time will allow one to understand how these two systems are inter-related. Also, although it is firmly established that local inflammation in the lung is associated with ACPA production, it is uncertain yet whether biomarkers of mucosal inflammation, such as fecal calprotectin, or responses to bacteria, such as increased expression of RegIII proteins, are altered in At-Risk subjects. Beyond these considerations, although studies of single mucosal sites have been performed in At-Risk subjects, a more comprehensive approach to simultaneously sampling multiple mucosal sites would allow one to understand whether changes are unique to one site or are shared across multiple sites, and then whether one site is sufficient to lead to systemic spread of ACPA generation. To address inter-relationships between local mucosal findings and systemic immune changes, additional studies of T cell antigen-specific or innate immune reactivity should be evaluated, both systemically and at mucosal sites. To further expand our understanding, a determination of whether other RA-related autoantibodies, including AMPA, are similarly generated locally in the mucosa as part of the hard wired innate immune system and then spread systemically should be undertaken. Likewise, understanding how systemic immune and metabolomics changes related to mucosal inflammation and ACPA production will provide additional insights. And finally, it would be important to determine whether this concept of “hard-wired mucosal autoimmunity” playing important roles in normal mucosal immune responses is relevant to the initial pre-clinical stages of other autoimmune diseases, such as SLE, where antibodies to intracellular proteins are prominent and it is uncertain as to why these intra-cellular antigens that are released during normal inflammatory processes are “chosen” as targets of autoantibodies in these conditions.

Conclusions

Prior to the onset of the first clinical signs and symptoms of seropositive RA, most patients will have passed through a prolonged period of serologic RA-related autoantibody positivity. Herein the current understanding of the relationships during this pre-clinical period between mucosal alterations, including dysbiosis and inflammation, and the initiation of local and systemic ACPA production has been reviewed. Mucosal ACPA production in the lung, and likely other sites, is associated with the presence of local inflammation and elevated NET formation. Mucosal IgA ACPA production appears to be a hard-wired and common phenotype, suggesting that this factor plays an important role in the local protective immune response outside of its eventual causal relationship with inflammatory arthritis. There are many potential factors in the mucosa, as well as systemic alterations, that could influence both the local initiation and systemic expansion of ACPA expression. Further study of these processes should provide both additional insights into very early disease pathogenesis in RA as well as the identification of novel therapeutic strategies.

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Glossary terms

Dysbiosis	A general term used to indicate a change in the normal bacterial ecology, usually with a potential for association with a disease state
Shared Epitope	A set of HLA antigens that are preferentially found in patients with RA, and which are characterized by conserved amino acids within the peptide-binding groove
Epitope spreading	A process whereby the epitopes (target antigen shapes or sequences recognized by B and T cell receptors) that are distinct from and non-cross-reactive with an earlier epitope become major targets of an ongoing immune response
Avidity maturation	A process whereby the accumulated strength of multiple affinities of individual non-covalent binding interactions between antigens and antibodies increases over time
NETosis	A unique form of neutrophil extracellular trap (NET)-associated cell death characterized by nuclear condensation and extrusion of chromatic and granular contents in a manner especially conducive to capturing pathogens
Molecular mimicry	The development of autoreactivity commonly considerd to be through the recognition of a microbial or other foreign pathogen peptide that is structurally similar to self-antigen

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Findings in At-Risk Populations Supportive of the Mucosal Origins Chronic Inflammation Hypothesis

Serologic:

- Elevated levels of, and expanded epitope recognition by, IgA and IgG ACPA
- Elevated levels of cytokines and chemokines
- Elevated CRP
- Elevated antibodies to *P. gingivalis*

Peripheral Blood Cell Phenotypes:

- Elevated proportion of circulating IgA-encoding plamablasts
- Elevated citH3, stimulated IL-17 and IL-2 levels, and decreased stimulated ATM and PFKFB3 levels

Findings at Mucosal Sites:

- Lung High Resolution Computerized Tomography demonstrating small airways diseases
- Elevated RA-related autoantibodies in sputum of At-Risk individuals
- Hypercellularity and increased NET remnants in sputum of At-Risk individuals
- Periodontal production of ACPA in patients with periodontitis
- Cervicovaginal ACPA production

Key Points

Patients who eventually develop seropositive RA pass through a period of RA-related autoantibody positivity that is associated with elevated cytokines and chemokines

There are multiple means by which mucosal processes can influence the development of systemic immunity and autoimmunity

Individuals who are at high risk for the future development of RA demonstrate evidence of chronic systemic and mucosal inflammation

Rather than reflecting a loss of citrullinated antigen tolerance, evidence suggests that IgA ACPA are normally produced locally, and a systemic IgG response results from loss of externally focused compartmentalization

Ongoing studies are linking the development of mucosal dysbiosis, inflammation and autoantibody production to the next stages of development of systemic autoimmunity

Novel autoimmune-promoting processes are likely to be identified that act primarily, or exclusively, in the pre-clinical period of RA and could be the targets for new prevention strategies

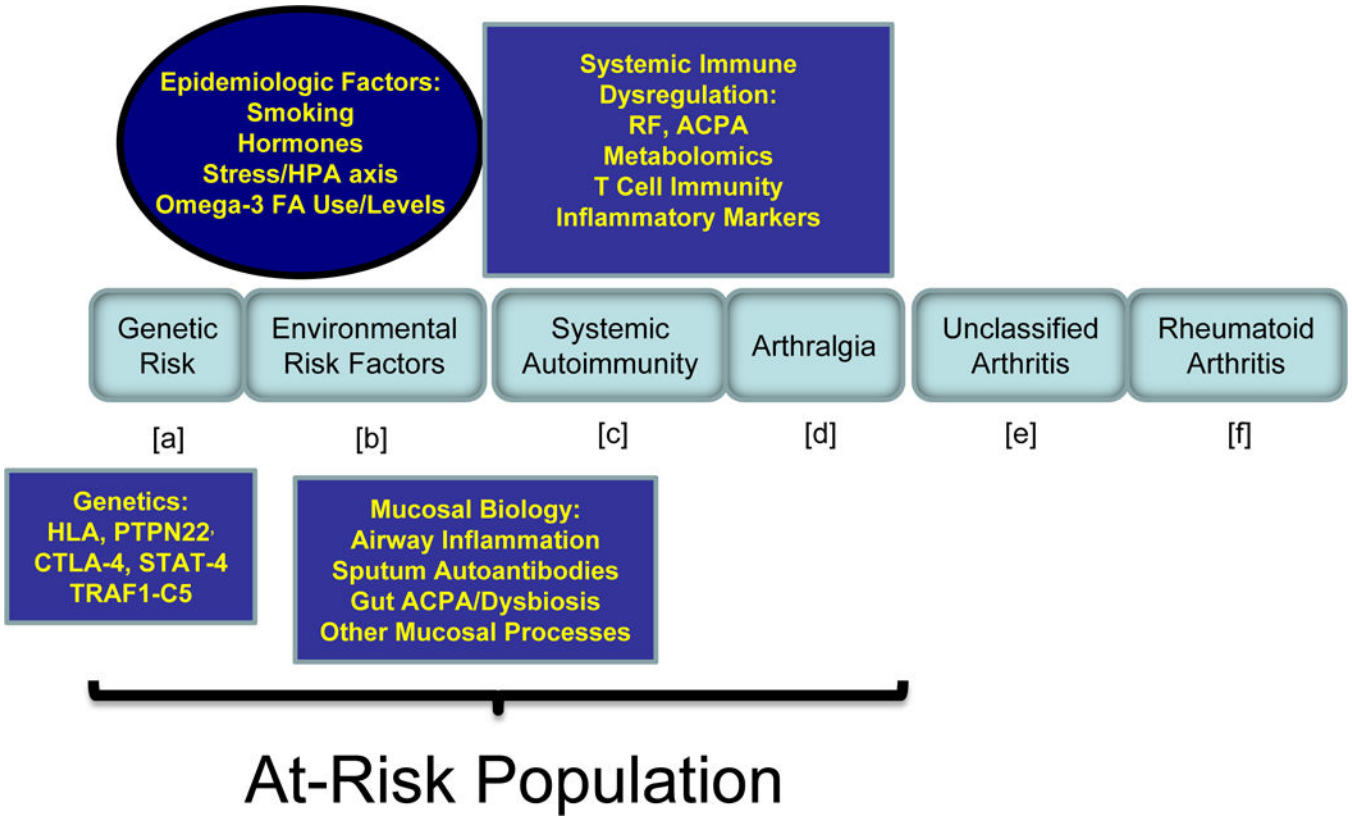

Figure 1. Biomarker studies relevant to pre-clinical RA pathogenesis.

Shown in the middle are EULAR recommendations for definition of disease stages (a – f) in the natural history of RA, with the pre-clinical stages bracketed. Boxes above and below the natural history stages include experimental approaches that are discussed herein and utilized through the pre-clinical period to understand the causal drivers of pre-clinical disease. Study populations include retrospective serum samples in addition to analyses of biomarkers in At-Risk populations.

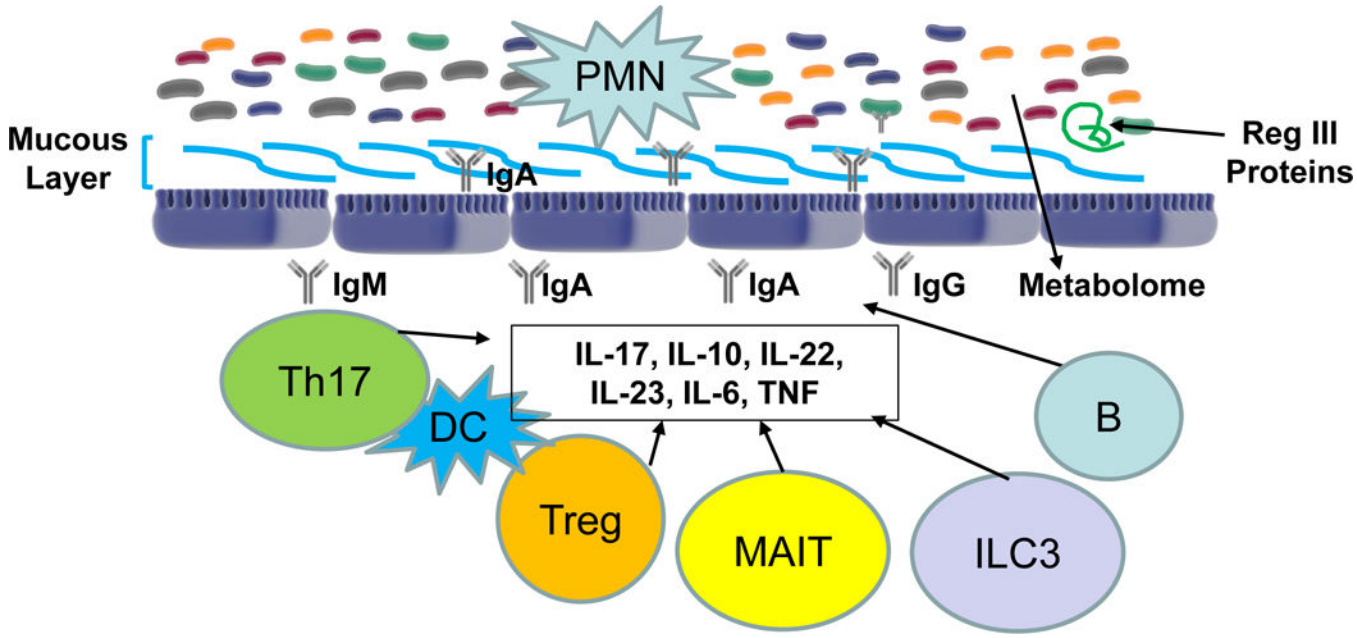

Figure 2. Local immune system and microbial factors that interact in the mucosal environment.

Illustrated are components of the bi-directional regulation between the luminal microbiome and the cells within the mucosa itself, with several cell types contributing individually to produce local cytokines. Specific examples are described in the text, but for example MAIT cells produce IL-17 while ILC3 are responsible for elaborating IL-22. PMN: polymorphonuclear neutrophils.

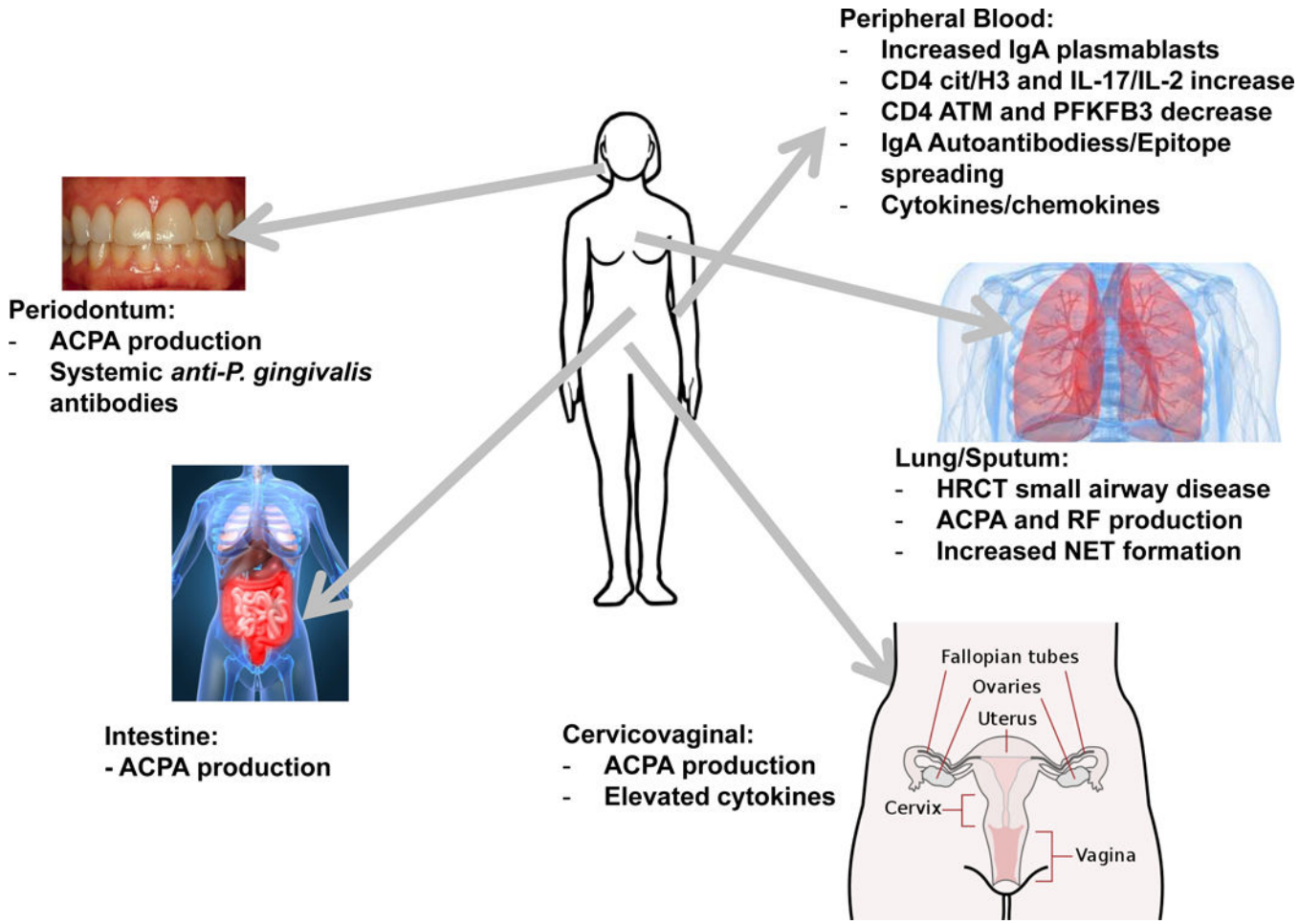

Figure 3. Representations of immune alterations in At-Risk individuals.

Studies of At-Risk individuals demonstrate immune alterations, both systemically and within local mucosal sites, consistent with the presence of chronic mucosal inflammation. Shown are findings found in these At-Risk populations. Not included are findings from patients with classified RA, as it is unknown as to whether those factors play a role in pre-clinical RA.

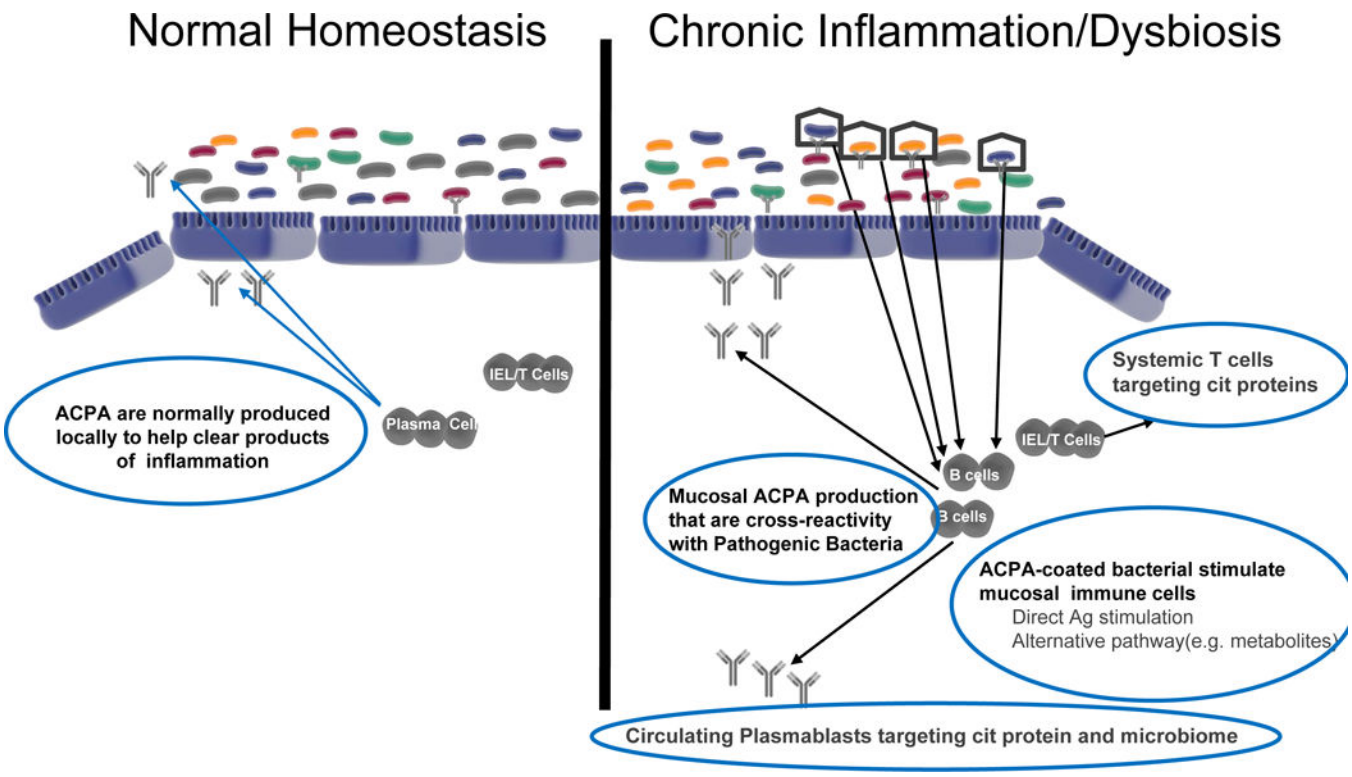

Figure 4. Loss of the mucosal firewall allows systemic spread of ACPA response.

Hypothetical model for RA initiation as chronic mucosal inflammation and then transition to systemic autoimmune disease, focusing on the potential roles for normal and dysregulated local mucosal ACPA generation. Depicted at left is the reversible production of IgA ACPA in individuals as part of normal surveillance or who have transient mild inflammation. This process is common and part of normal homeostasis at the mucosal surface. At right is the postulated status of individuals in the pre-clinical RA state where persistent inflammation leads to increased ACPA production and systemic spread of an autoimmune process through multiple potential processes described herein. The drivers of this local and then systemic process could include cytokines, metabolomic changes, cross-reactive ACPA with bacterial antigens, or the presence of other antigen-specific cells that are expanded. Regardless of the mechanisms involved, these antibodies and cells begin to circulate as a reflection of the chronic inflammation and are detectable through serologic and other biomarker studies.

Table 1

Environmental factors affecting the natural history of RA

Environmental exposures and effects that have been shown to impact the total and/or stage-specific course of RA development.

Environmental exposure	Patients with RA/Predominant Direction of Risk	Subjects At-Risk of developing RA and/or with pre-clinical RA/Predominant Direction of Risk
Smoking	Yes/Increase	Yes/Increase
Omega-3 FA use	Yes/Decrease	Yes/Decrease
Oral contraceptive use	Yes/Decrease	Yes/Decrease
Breast-feeding	Yes/Decrease	Uncertain
Earlier age at menarche	Yes/Variable	Uncertain
Body mass index	Yes/Increase	Yes/Increase
Decaf coffee consumption	Yes/Increase	Uncertain
Silica dust, solvents, mineral oil, textile dust	Yes/Increase	Yes/Increase
Physical workload	Yes/Increase	Uncertain
Proximity to road/Air pollution	Yes/Increase	No
UV Light	Yes/Decrease	Uncertain
Alcohol	Yes/Decrease	Uncertain