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Review

The pathogenesis of rheumatoid arthritis

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SUMMARY

Significant recent progress in understanding rheumatoid arthritis (RA) pathogenesis has led to improved treatment and quality of life. The introduction of targeted-biologic and -synthetic disease modifying anti-rheumatic drugs (DMARDs) has also transformed clinical outcomes. Despite this, RA remains a life-long disease without a cure. Unmet needs include partial response and non-response to treatment in many patients, failure to achieve immune homeostasis or drug free remission, and inability to repair damaged tissues. RA is now recognized as the end of a multi-year prodromal phase in which systemic immune dysregulation, likely beginning in mucosal surfaces, is followed by a symptomatic clinical phase. Inflammation and immune reactivity are primarily localized to the synovium leading to pain and articular damage, but is also associated with a broader series of comorbidities. Here, we review recently described immunologic mechanisms that drive breach of tolerance, chronic synovitis, and remission.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic destructive inflammatory synovitis, accompanied by wider clinical sequelae including comorbidities particularly affecting systemic bone, vasculature and metabolic function, and cognition. RA can serve as a prototypic autoimmune disease in which a preclinical phase, without clinical detection is preceded by years of clinical onset of disease (Figure 1). Significant progress in RA management in the last two decades has resulted in two significant advances, which in turn have driven improvements across the wider immune-mediated inflammatory disease spectrum. First, the strategic approach to care moved from late-stage therapeutic intervention to early commencement of disease modifying anti-rheumatic drugs (DMARDs), in turn used in a disease activity targeted manner (treat-to-target). Second, immunologic advances since the identification of immune mechanisms involved in the disease pathogenesis led to the introduction of the introduction of tumor necrosis factor (TNF) inhibitors, followed thereafter by application of interleukin-6 receptor (IL-6R) inhibitors, checkpoint modifiers (Abatacept), and B cell depletion (Rituximab). Later introduction of key signal transduction pathways, specifically Janus Kinase (JAK) inhibitors, has further expanded the armamentarium, offering at least comparable efficacy to biologic interventions with oral bioavailability and potential superiority in some trials. These approaches, taken together, substantially reduced the morbidity and mortality associated with RA and in current practice enable the achievement of drug-maintained remission. However, RA is a highly heterogeneous disease and considerable unmet needs remain. These include partial and non-response to treatment in many patients, failure to achieve immune homeostasis or indeed drug free remission, and no capacity to repair tissues once damaged. Deeper understanding of RA pathogenesis will be critical to solve disease heterogeneity and to address these outstanding issues. We previously extensively reviewed the pathogenesis of RA (Firestein and McInnes, 2017) and now provide an update that helps inform our current understanding of pathogenesis.

GENE-ENVIRONMENT-MICROBIOME INTERACTIONS DRIVE DEVELOPMENT OF RA

Risk factors for RA development, severity, and progression include a strong genetic component. The most important risk is located within the class II major histocompatibility (MHC) locus. HLA-DR4 is found in 70% of RA patients when compared to 30% of control subjects. The shared susceptibility epitope (SE) is located at the amino acid 70–74 position of the third hypervariable region of the DRβ chain (Weyland et al., 1992). The distinct amino acid position of the DRβ chain apart from the SE explains most association between MHC and seropositive RA (Raychandhuri et al., 2012), since it affects the avidity by which arthritogenic antigens are bound. On this genetic background, post-translational modification, particularly citrullination, of a range of self-proteins creates altered self-antigens that apparently act as prime generators of the autoimmune $CD4^+$ T cell response in anti-modified peptide antibody $(AMPA)^{pos}$ RA patients.

Citrullination, resulting from the conversion of arginine into citrulline by peptidyl arginine deiminases (PADs), is a ubiquitous phenomenon in mammals that can be induced by environmental factors such as smoking (Klareskog et al., 2006). Cigarette smoking is the strongest risk factor for RA development (Malmstrom et al., 2017). Smoking acts as an epigenetic and mucosal

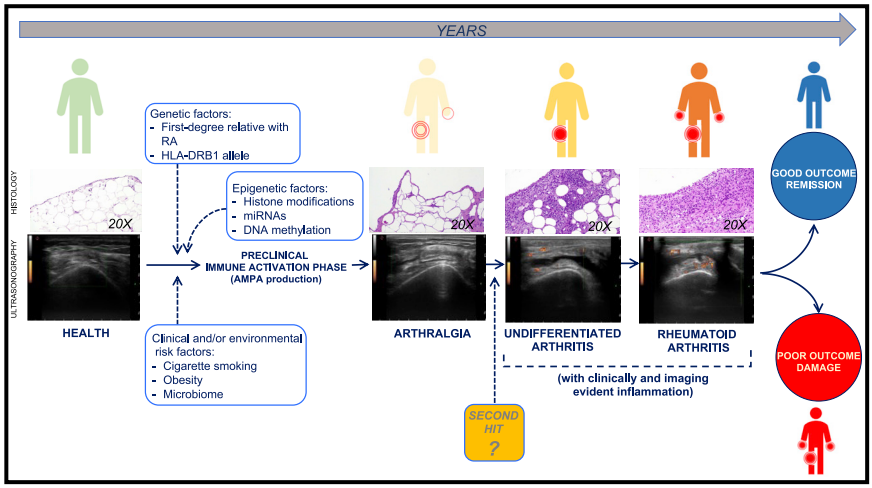

Figure 1. Sequential events of the disease trajectory in rheumatoid arthritis

Rheumatoid arthritis (RA) is the prototype of autoimmune disease in which a preclinical phase, without clinical detection, is preceded by many years clinical onset of disease. The schematic depicts the sequence of events leading to the development of clinically detectable RA. A pre-RA phase comprises early generation of autoantibodies (AMPAs) that can bind post-translationally modified self-proteins, particularly via citrullination. This can be facilitated by environmental risk factors as cigarette smoking and/or mucosal microbiota disturbance. This is followed by amplification of the range of specificities of AMPA and by the elaboration of cytokines and chemokines, complement, and metabolic disturbance in at risk population in the months prior to clinical development of disease. A transition event that requires a 'second hit', as yet poorly understood permits the development of clinically and imaging-detected synovitis. The latter is characterized by synovial hyperplasia and increased Power Doppler signal (related to increased vascularity) and histologically by lining layer hyperplasia, increased stromal density and immune cells infiltration leading to articular damage. Synovial tissue features at disease onset might inform about the prognosis and treatment response of RA patients. Pictures show examples of Haematoxylin an Eosin staining of US-guided minimally invasive synovial tissue biopsies of the knee (20× magnification) taken from patients fulfilling each disease phase criteria at the SYNGem Biopsy Unit of the Fondazione Policlinico Universitario A. Gemelli IRCCS in Rome (Italy) with the correspondent Gray scale and Power Doppler findings.

microbiota modifier and exerts its effect in RA mainly in individuals carrying HLA-SE alleles (van der Helm-van Mil et al., 2007; Michou et al., 2008; Ishikawa et al., 2019). HLA-SE and smoking seem to act in distinct pre-clinical stages of RA; smoking increases the risk of anti-citrullinated peptide autoantibodies (ACPA) development, whereas HLA-SE does not associate with ACPA positivity per se in the general population but increases the likelihood to progress toward inflammatory arthritis (Wouters et al., 2022). Smoking-related risk prompted extensive evaluation of the role played by mucosal events in disease etiology, particularly the lung.

The pathogenesis of RA might begin at the mucosal level prior to transition toward synovial involvement (Holers et al., 2018). Epidemiological studies and high-resolution chest CT scans of pre-RA and early RA patients reveal an association between smoking, bronchiolar mucosal abnormalities, and the development of RA. ACPA, and RF can be produced by inducible bronchus-associated lymphoid tissue (iBALT) in RA patients. Microbiome studies of bronchoalveolar lavage fluid (BALF) of early RA patients reveal that lung microbiota was less diverse and abundant than in healthy controls. In particular, the genus *Pseudonocardia* was over-represented in BALF of early RA patients, correlating with disease activity and presence of bone erosions (Scher et al., 2016). Definitive data concerning BALF microbiota composition in individuals at risk of RA development are sparse. Analysis of sputum reveals that at-risk sputum is enriched for RA-related autoantibodies even in the absence of serum ACPA and RF (Willis et al., 2013). Mucosal-serum ratios suggest that the lung represents a primary site of autoantibody production. Production of local IgA ACPA has been associated with neutrophil and macrophage enrichment and with formation of endogenous neutrophil extracellular traps (NET) (Demoruelle et al., 2017).

Cross-sectional studies comparing ACPA reactivities in sputum of at-risk populations and early RA show an increase of sputum-derived ACPA reactivities in the latter suggesting that epitope spreading can occur in the lung (Demourelle et al., 2018). A cross-sectional study comparing different categories of patients from at-risk individuals to patients with active RA and controls has revealed that in at risk subjects, independent from smoking, inflammatory cytokines (IL-6, TNF, and IL-1) and complement pathway proteins are associated with sputum IgA ACPA through cit-Histone3 containing NET remnants (Okamoto et al., 2022). Moreover, sputum macrophages from at-risk subjects and RA patients have reduced endocytic function suggesting that NET formation and decreased NET clearance might contribute to the high concentrations of NET remnants associated with sputum IgA ACPA. Definitive proof of NET remnants ingestion by macrophages is still needed (Okamoto et al., 2022). The prognostic role of such findings should be confirmed by future studies enrolling cohorts of at-risk individuals followed longitudinally through to RA development.

The mechanisms by which oral mucosal tissues respond to environmental cues are still largely unknown. Recently, a comprehensive cell atlas of the human oral mucosa showed that the healthy oral mucosa is generally similar to other human barrier tissues in terms of epithelial, endothelial, immune, and stromal cell composition (Winslow Williams et al., 2021), aside from enrichment in neutrophils. During inflammation—e.g., periodontitis disease, which leads to the destruction of tooth-supporting structures, including bone (Potempa et al., 2017)—there is a profound gingival inflammatory infiltrate with an inflammatory shift in epithelial and stromal cells. The latter shows upregulation of pathways related to cell adhesion for lymphocytes (*CCR7*, *CCR6*, *FPR1*, *LRP1,* and *C3AR1*) and neutrophils (*CXCL8*, *CXCL1*) and chemokine signaling suggesting an active role in recruitment of immune cells. Analysis of the integrated scRNAseq datasets from periodontitis versus healthy tissue demonstrates an expansion of T lymphocytes, neutrophils, and plasma cells. Thus, the cellular machinery needed for the loss of immunological tolerance could be contained in gingival mucosa to drive local auto-antibody production in a genetically

predisposed individual. Moreover, smoking is also a risk factor for development of periodontitis (Jepsen et al., 2017; Kharlamova et al., 2016).

Several studies potentially link periodontal disease and RA (Potempa et al., 2017). *Porphyromonas gingivalis* can induce citrullination through peptidylarginine deiminase 4 (PAD4) leading to post-translational modification generating novel autoantigens. A cross sectional characterization of the oral microbiome in ACPA+ at-risk individuals revealed difference in nearly 35%–40% species composition of the subgingival plaque compared to healthy controls. *Porphyromonas gingivalis* has higher relative abundance in ACPA+ at-risk subjects supporting a potential role in RA initiation (Cheng et al., 2021). Other infectious agents could drive molecular mimicry promoting breach of immunological tolerance. *Glaesserella parasuis* causes severe swine arthritis and shares sequence similarity with residues 261–273 of collagen type 2 ($Coll_{261-273}$), a possible autoantigen in RA. A cross-sectional study including RA patients and patients with undifferentiated peripheral inflammatory arthritis (UPIA) has found *Hps* DNA in nearly 60% of the tooth crevicular fluid of RA patients, double that in healthy controls. Interestingly, anti-*Hps* IgM and IgG have been found in serum of RA patients and directly correlate with disease duration. *In vitro* stimulation of HLA-DR4+ derived PBMCs with live *Hps* or with *Hps* virulence-associated trimeric autotransporter peptide ($VtaA10_{755-766}$), which is homologous to human $Coll_{261-273}$ elicit IL-17A release (Di Sante et al., 2021). There is still insufficient understanding about the longitudinal effects of persistent periodontal infection across different phases of RA in terms of treatment response, as well as of remission maintenance or flare. For example, data from the ESPOIR cohort of patients with established RA has shown that good oral hygiene significantly reduces the load of periodontal pathogens without improving disease activity (Mariette et al., 2020).

The hypothesis that oral or intestinal microbiota composition plays a role in RA pathogenesis is further supported by the finding that dysbiosis and intestinal inflammation develop before clinical arthritis onset and persist thereafter. Antibiotic treatment before collagen induced arthritis (CIA) induction has a beneficial impact on disease severity and pro-inflammatory mediator and anti-collagen antibodies release (Jubair et al., 2018). Stool samples of patients with new onset RA show expansion of *Prevotella copri* and a reduction of *Bacteroides* compared to RA patients with established disease or other chronic joint inflammatory disease (Chen et al., 2016; Scher et al., 2013). Fecal microbiota transfer from RA patients to mice with the defective ZAP70 kinase significantly increases their susceptibility to spontaneous arthritis development (Maeda et al., 2016). In contrast, *Prevotella histicola* exerts a beneficial effect *in vivo* in a murine model of chronic inflammation (Mangalam et al., 2017). Tandem mass spectrometry on RA synovial tissue, fluid, and PBMCs has revealed a HLA-DR presented *Prevotella*-specific peptide (Pc-p27) that induces a T helper-1 (Th1) response in RA patients, and against which a specific antibody response is detected only in RA patients (Pianta et al., 2017). Interestingly, a cross-sectional analysis of fecal microbiota has shown an enrichment of *Prevotella spp*. In individuals at pre-clinical RA stage compared to simple first-degree relatives (Alpizar-Rodriguez et al., 2019). A systematic metagenomic shotgun sequencing and metagenome-wide association study (MGWAS) of fecal, dental, and salivary samples from RA patients and healthy controls, has revealed concordance among gut and oral microbiomes suggesting overlap between different body sites. Distinct gut and oral microbiome signatures have been related to clinical parameters and partially normalized after pharmacological treatment. In particular, all three sites show enrichment of *Lactobacillus salivarius*, mainly in very active RA patients (Zhang et al., 2015).

Obesity might also play a role in RA initiation, development, and progression since elevated BMI is an established disease risk factor. In theory, obesity could modify other risk factors: e.g., via promoting dysbiosis, by promoting mucosal immune dysregulation via dietary elements (e.g., short chain fatty acids), or via amplification of inflammation via adipocytokine release. Mechanisms are however poorly characterized. Mice fed a high-fat diet exhibit earlier onset of collagen-induced arthritis compared to regularly fed mice. Arthritis resolution is accelerated in lean compared to obese mice (Kim et al., 2016). In RA, synovial membrane inflammatory composition and transcriptome are contingent with overweight and obesity status only if naive to treatment, at disease onset, and at the time of sustained clinical and ultrasound remission (Alivernini et al., 2019). Myeloid cells in RA adipose tissue biopsies are increased in number and associated with systemic inflammation, autoimmunity, and whole-body insulin resistance (Giles et al., 2018). In this context, the Nurses' Health Study (NHS) has elicited an association between active and passive smoking (Liu et al., 2019; Yoshida et al., 2021), dietary habits (Sparks et al., 2019), obesity (Lu et al., 2014b), and RA development, strongly supporting their role in disease pathogenesis.

Taken together, these findings support the notion that a multifactorial pathogenetic cascade including genetic, environmental, and microbiome interactions might drive the development of RA.

ANTIBODIES AGAINST POST-TRANSLATIONALLY MODIFIED PROTEINS PRECEDE CLINICAL RA

The delay between breach of immunological tolerance and clinical manifestation of disease is a common finding in human autoimmune diseases, such as type 1 diabetes mellitus (T1DM) (Kulmala et al., 1998). The earliest manifestation of immune dysregulation in RA is detection of circulating ACPAs, associated with elevated circulating cytokines. Healthy individuals may develop low titers of ACPA associated with concomitant risk factors, e.g., smoking and periodontitis. In the absence of HLA-DR SE, these generally will not promote disease (Padyukov et al., 2004; Koning et al., 2015; Alpizar-Rodriguez et al., 2017; Terao et al., 2015). With HLA-SE, the presence of ACPA is associated with development of disease and thereafter, a more aggressive clinical course, with increased propensity to articular damage. ACPA directly activates myeloid cells *in vitro* via direct interaction with citrullinated membrane proteins, and adjacent FcR engagement to increase cytokine release. However, they fail to induce arthritis after passive transfer *in vivo*. ACPA can exacerbate pre-existing joint inflammation, however, if co-administered with other pathogenic antibodies (anti-collagen) or additional boosters (LPS) (Titcombe et al., 2018; Uysal et al.,

2009; Ozawa et al., 2020). These findings support the notion that ACPA needs additional triggers to exert pathogenetic effects.

Studies of ACPA glycosylation suggest differential pathogenic or protective actions exerted by distinct sugar residue modifications. The amino acid sequence of the Fc region and Fc-linked carbohydrate structures within the heavy chain, in close proximity to FcR and complement system engagement, influence antibody effector functions (Scherer et al., 2010). Increase of agalactosylated IgG glycans is considered a hallmark of aging and other chronic inflammatory diseases (Gudelj et al., 2018), while sialylation of the IgG Fc domain was found to impair complement dependent cytotoxicity (Quast et al., 2015). ACPA IgG1 and total IgG1 from serum of arthralgia individuals at risk of RA development show similar Fc glycosylation pattern, whereas 3 months prior to diagnosis and progressively until the time of arthritis onset, ACPA exhibit decreasing galactose residues in parallel with increasing systemic inflammatory parameters (Rombouts et al., 2013). Conversely, structural analysis of ACPA IgG shows that the (hyper) variable domains of ACPA are characterized by a high frequency of N-glycan residues, which impact binding avidity to citrullinated antigens (Rombouts et al., 2016) and their presence predicts the development of RA, suggesting a direct pathogenetic role (Hafkenscheid et al., 2019).

Antibodies against other post-translational modifications including carbamylation or acetylation are also detected in RA (Trouw et al., 2017). Homocitrullination (carbamylation) entails modification of lysine residues. Homocitrulline residues are localized at different positions within the protein structure, leading to the development of different self-antigens. Anti-carbamylated peptide (anti-CarP) can be detected years before clinical onset and their presence is a risk factor for RA development. Anti-CarP antibody positivity does not fully overlap with ACPA since they occur in 16%–20% of seronegative RA patients. Whereas their antigenic specificity increases in proximity to clinical disease onset, their presence is not associated with HLA-SE (Brink et al., 2015; Shi et al., 2014). In mice, anti-CarP antibodies are inducible after immunization with carbamylated antigens (Cantaert et al., 2013); however, their induction is not able to induce arthritis development. In a CIA mouse model, the production of anti-CarP antibodies precedes the arthritis development and enhances its severity (Stoop et al., 2014). Production of anti-CarP antibodies in animal arthritis appears to reflect the presence of non-specific inflammatory triggers, e.g., infections. Isolation of ACPA by affinity purification through citrullinated antigens recover a pool of antibodies showing cross-reactivity toward carbamylated antigens suggesting the existence of a broad antigenic repertoire able to stimulate B cells toward post-translationally modified proteins (Reed et al., 2016, 2020). Anti-acetylated protein antibodies are also detected in nearly 30%–40% RA patients (Juarez et al., 2016; Kampstra et al., 2019), although their functional role is less well understood.

ACPAs are apparently part of a broader spectrum of autoantibodies that target post-translational modification collectively termed AMPA. Although multiple different AMPA can be simultaneously present in an RA patient, their role in combination for diagnosis, prognosis and pathogenicity, remains uncertain. Recently, a study of naive-to-treatment early RA patients, has shown that triple-positive patients had more radiographic progression over 12 months than did single-specificity positive patients. Radiographic progression in the single-positive group was similar to that of patients who were seronegative for all AMPAs, suggesting that the effect on radiographic progression of being ACPA-positive is restricted to those patients who also have other AMPAs (Nijjar et al., 2021). Whether this reflects the specific functional interaction of distinct AMPAs or rather that the breadth of AMPAs reflects the depth of immune dysregulation is unclear.

T CELLS LIKELY PLAY A ROLE IN VERY EARLY RA PROGRESSION

Although multiple studies have shown the importance of thymic T cell selection in autoimmunity (Sakaguchi et al., 2003; Kishimoto and Sprent, 2001), the potential role of the HLA risk alleles in shaping the T cell repertoire during thymic selection has recently emerged. A strong association between HLA-DRB1 amino acid position 13 and CDR3, conferring antigen recognition on T cell receptors was found in RA and other autoimmune diseases, strongly supporting the central hypothesis in breach of immune tolerance in human RA (Ishigaki et al., 2022). During pre-clinical RA, ACPA isotype diversity and titer increase is likely driven by $CD4^+$ T cells supporting affinity maturation in germinal centers (Koning et al., 2015). Healthy individuals carrying HLA-DR4 and asymptomatic first-degree relatives of RA patients are characterized by specific telomere damage and abnormal T cell differentiation (Schonland et al., 2003; Waase et al., 1996). Moreover, peripheral-blood-derived $CD4^+$ T cell phenotype composition across the RA disease spectrum has found that individuals at risk of RA development, who progressed eventually toward clinically defined inflammatory arthritis, express an enriched population of inflammation-related cells (T cell subset expressing both naive and memory differentiation markers) and a significant reduction of naive and regulatory T (Treg) $CD4^+$ cells, when compared to non-progressors (Ponchel et al., 2020).

Systematic immunohistochemical analysis of synovial tissue biopsies obtained from AMPA- and RF-expressing subjects indicates that proportions of macrophages, T and B lymphocytes, and synovial fibroblasts (FLS) are similar between AMPA-positive arthralgia individuals and controls regardless of subsequent clinical outcome (van de Sande et al., 2011). These data suggest that an additional biological signal required for the development of clinical arthritis might act on the qualitative composition of resident synovial inflammatory cells or stromal cells. Advances in multiparametric analysis, including flow cytometric and computational tools, have allowed characterization of a polyfunctional T cell response in the preclinical phase of RA (Floudas et al., 2022). Synovial tissue of individuals at risk of arthritis development is characterized by enrichment of signaling pathways involved in T cell activation and polarization leading to increased $CD4^+$ T cell polyfunctionality. A $CD4^+CD8^{dim}$ T cell population has been identified as highly polyfunctional, metabolically primed, with high proliferative capacity, and, importantly, with limited resistance to suppression by autologous Treg cells when compared to their $CD4^+$ T cell counterpart (Basdeo et al., 2015). Similarly, $CD4^+CD8^{dim}$ T cells have been found in RA peripheral blood (Washbisch et al., 2014; Quandt et al., 2014;

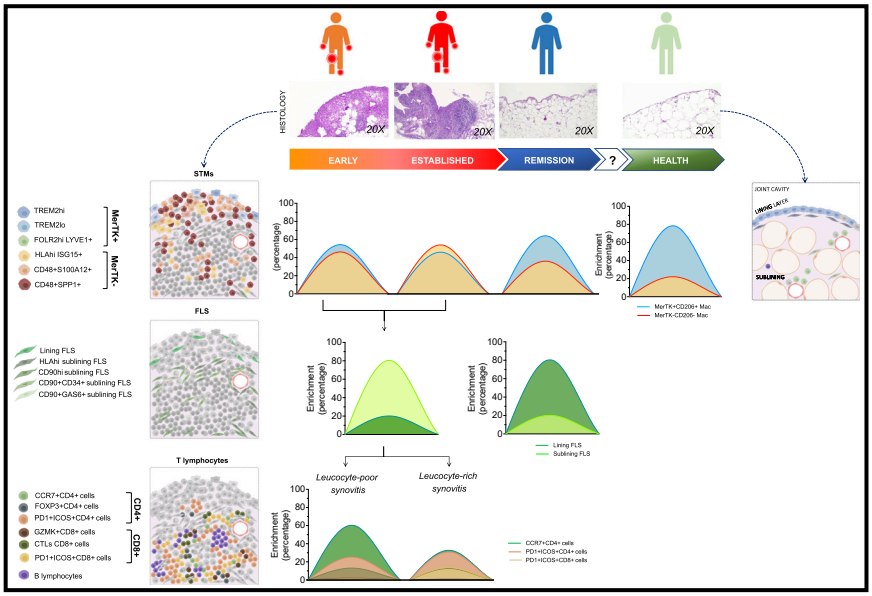

Figure 2. Schematic of synovial macrophages, fibroblast like synoviocytes, and T lymphocyte clusters identified using single-cell RNA sequencing experimental approaches in tissues obtained from healthy subjects and patients with RA across different disease phases

The synovial membrane is the target tissue of rheumatoid arthritis inflammation, characterized in health by a thin lining layer made of resident macrophages and fibroblast like synoviocytes attached to a sublining layer enriched of adipocytes, vessels and few immune cells. During RA inflammation, synovial tissue is characterized by lining layer and stroma cells hyperplasia, and an influx of immune cells from the circulation leading to a variable enrichment of myeloid and lymphoid microanatomical organization. In the last years, single-cell RNAs sequencing experiments identified the heterogeneity of resident synovial tissue macrophages (STMs) from healthy subjects and patients with RA. Based on the expression of tyrosine kinase MerTK, STMs can be distinguished into 2 main populations with inflammatory phenotype (MerTK-CD206-) or homeostatic/anti-inflammatory properties (MerTK+ CD206+), which are differentially expanded in RA patients in relation to the disease state. In particular, synovial tissue of RA patients in sustained remission is enriched of MerTK+ clusters whose rate is an independent predictor of remission maintenance after treatment modification. Moreover, different clusters of fibroblast-like synoviocytes (FLS) have been identified in the synovial tissue of RA patients with distinct location-dependent transcript whose enrichment is contingent with RA phase. Finally, different clusters of synovial tissue T-lymphocytes have been identified with distinct inflammatory function and differentially distributed based on the synovial tissue histology in RA patients. The enrichment graphs were created using enrichment rates from Alivernini et al. (2020) (STMs and FLS in synovial tissue of active RA and RA in sustained remission) and from Zhang et al. (2019) (T-lymphocytes in synovial tissue of active RA based on leukocytes enrichment).

Bohner et al., 2019). Although $CD4^{+}CD8^{dim}$ T cells may comprise thymic escapees able to give rise to autoreactive T cells, their ontogeny is still controversial (Overgaard et al., 2015) and longitudinal synovial biopsy studies are necessary to define their potential dynamic pathogenetic role.

Since RA-specific autoantibodies can be detected years before the onset of joint symptoms in individuals without evidence of increased synovial inflammation, pathogenetic mechanisms acting outside the synovial compartment are likely responsible for the initial changes leading to the development of RA. As described, this may be the pulmonary mucosa. However, a pilot study of draining lymph-nodes of individuals at risk of RA development (ACPA and/or RF+) compared to early RA or healthy controls reveal increased immune cell activation within lymph nodes of early RA patients as well as in ACPA and/or RF+ individuals at risk of developing RA (van Baarsen et al., 2013). CXCR5+ follicular T helper and $CD19^{+}$ B cells are expanded in lymph nodes of pre-arthritis patients and of patients with early RA (Cuo Anang et al., 2022).

IMMUNE STROMAL CELL INTERACTIONS DRIVE CHRONICITY IN RA SYNOVITIS

Resident cells of the synovial tissue, namely synovial fibroblast-like synoviocytes (FLS) and macrophages, represent key components of the joint microenvironment contributing to niche homeostasis and inflammatory disruption (Alivernini et al., 2020; Buckley, 2011; Buckley and McGettrick, 2018). A rich history in synovial tissue analysis by immune-histology, transcriptomics, and *ex vivo* culture and functional evaluation has clearly defined central roles, especially for these two cell types (Nygaard and Firestein 2020). More recently, advanced tissue analytic methodologies have shed light on significant new insights. Multimodal profiling enabling simultaneous analysis of multiple cellular characteristics has been developed (Stuart and Satija, 2019) to compare scRNA sequencing with another cellular characteristic (e.g., DNA sequence, protein expression, or epigenetic profile). Recent studies investigating the stromal and myeloid compartments of the synovial tissue of RA patients reveal a significant heterogeneity in terms of phenotype and cell functions in the stromal and macrophage synovial populations across the RA trajectory (Croft et al., 2019; Alivernini et al., 2020; Zhang et al., 2019). These cell types might predominate functionally over T cells in mediating chronicity.

SYNOVIAL STROMAL CELLS EXHIBIT DIVERSE PHENOTYPIC PROPERTIES

The synovial tissue forms a barrier between membrane and fluid compartment enriched in hyaluronic acid (HAS1) and lubricin (PGR4) (Smith, 2011; Smith et al., 2003; Rhee et al., 2005) whose function is mainly to reduce friction between cartilage surfaces (Rhee et al., 2005). Synovial tissue is divided into two distinct regions: (1) a continuous 1–3 cells thick FLS/macrophage lining layer and (2) a pauci-cellular underlining region (sublining layer) in which resident FLS and macrophages are sparsely distributed together with adipocytes, blood and lymphatic vessels and rare inflammatory cells (Smith, 2011; Culemann et al., 2019) (Figure 2). FLS serve in part the maintenance of synovial extra-cellular matrix (ECM) through production of matrix components (e.g. collagens, tenascin, laminin, proteoglycans) and enzymes

responsible for EMC degradation (i.e. matrix metalloproteinases, cathepsins, proteases).

Extensive and elegant studies over four decades have firmly established that FLS drive inflammation amplification and structural damage via production of chemokines, cytokines and lytic enzymes enhancing recruitment and activation of infiltrating immune cells together with promotion of bone erosion (Firestein 1996; Nygaard and Firestein 2020). Herein, we will focus on more recent studies. Two transcriptionally distinct fibroblast 'populations' have been identified in RA synovial tissue based on their tissue location, as $CD55^+$ FLS located in the lining layer and $CD90^+$ (THY+) located in the sublining layer. Further studies have described phenotypic differences between RA FLS populations analyzed from enzymatically digested synovial tissue specimens (Croft et al., 2019; Zhang et al., 2019; Mizoguchi et al., 2018). There are three different clusters of FLS with specific anatomical distribution: $CD34^-CD90^-$ FLS (expressing MMP1, MMP3, PRG4, and HAS1) are located In the lining layer, $CD34^+CD90^+$ FLS in proximity to the sublining vasculature, and $CD34^+CD90^-$ FLS with a wider distribution (Mizoguchi et al., 2018). The $CD34^+CD90^+$ FLS cluster is expanded in RA synovial tissue compared to OA and expression correlates with histologic synovitis severity. An integrated analysis of FLS heterogeneity combining scRNA sequencing transcriptomics and mass cytometry have revealed 3 distinct $CD90^+$ sublining fibroblast phenotypes. HLA-DRhi and $CD34^+$ cells are expanded in leukocyte-rich synovial tissues (Zhang et al., 2019). In particular, the RNA expression profile of HLA-DR+$CD90^+$ FLS suggests that these cells could be a source of IL-6 and potentially internalize, process and present antigens via HLA-DR. HLA-DR+$CD90^+$ FLS are characterized by a proinflammatory phenotype and can physically interact with immune cells, e.g., NK or $CD8^+$ T cells (Zhao et al., 2022). Immune-complex activated NK cells might therefore contribute to more severe disease. In addition, the expression of MHC-Class II on the FLS surface (Zhang et al., 2019) could support superantigen presentation by FLS (Carmona-Rivera et al., 2017). We note however that the degree of antigen presentation by FLS compared to other professional and non-professional antigen-presenting cells is unproven.

The stromal compartment of synovial tissue of RA patients with active disease highlights a role for fibroblast activation protein alpha (FAPα), which is absent during quiescent status. FAPα deletion in mice, abrogates inflammation and reduces tissue damage (Croft et al., 2019). The FAPα+$CD90^+$ sublining FLS population contains "populations" with apparently distinct effector functions. For example, a $CD34^+CD90^+$ FLS population in subliming perivasculature promotes release of pro-inflammatory cytokines and chemokines, e.g., CXCL12 and CCL2, thereby promoting immune cell homing toward the synovium (Croft et al., 2019). Furthermore, CD34+C3+ FLS undergo metabolic reprogramming after repeated inflammatory stimuli supporting a putative role in the switch from self-resolving toward chronic inflammation that characterizes RA synovitis (Friscic et al., 2021).

Single-cell RNA sequencing analysis also revealed positional identity within RA synovial tissue. Performing an unbiased trajectory analysis, location-dependent transcriptomic variation of FLS has been identified spanning lining layer to perivascular regions driven by the local microenvironment. In a free-floating, three-dimensional synovial tissue organoid system, endothelial cells provide a positional signal for FLS. NOTCH3 signaling controls such FLS positional identity. RA synovial tissue is enriched in NOTCH3+ FLS compared to OA and NOTCH3 blockade attenuates inflammatory arthritis after serum transfer from K/BxN mice (Wei et al., 2020). FLS might be therapeutically targeted via modulation of NOTCH3 signaling. This study however also highlights that these stromal cell populations are probably not true "cellular subsets" but rather reflect spatially determined effector phenotypes regulated by their cellular microenvironment.

There is growing evidence that fibroblasts might share transcriptional states across different tissues. In particular, the analysis of datasets from single-cell atlas projects—e.g., Adult Human Cell Atlas (AHCA) (He et al., 2020) and Tabula Sapiens (The Tabula Sapiens Consortium and Quake, 2021)—profiling tissue samples from multiple organs has revealed that fibroblasts from different tissues group together, suggesting that lineage contributes more to transcriptional identity than the tissue of origin. Moreover, the integrated analysis of scRNA-seq datasets of fibroblasts across healthy and diseased conditions suggests the existence of a pluripotent DPT+ pluripotent universal fibroblast cluster that is present in healthy and disease and might give rise to functionally distinct fibroblasts in different tissue niches (Buechler et al., 2021). Identification of the driving pathogenetic mechanisms for such a universal cluster might provide additional therapeutic targets.

Distinct transcription factors belonging to the *ETS* family might function as controllers of fibroblast polarization. In particular, the ETS family transcription factor PU.1 is overexpressed in fibroblasts of fibrotic organs but not by fibroblasts from inflamed RA synovial tissue. Its overexpression converts inflammatory into fibrotic fibroblasts (Wohlfahrt et al., 2019). More recently, ETS1 has been found to be a key regulator of the pathological tissue-remodeling programs of fibroblasts (Yan et al., 2022). ETS1 drives polarization toward tissue-destructive fibroblasts in arthritis via receptor activator of nuclear factor-κB ligand (RANKL) as well as metalloproteinases (MMPs). Fibroblast-specific deletion of ETS1 significantly improves bone and cartilage damage in arthritis model (Yan et al., 2022). Co-expression analysis has shown that fibroblasts express either PU.1 or ETS1 but are unlikely to express both factors together, likely due to the cell type-selective expression of miR-155 (Wohlfahrt et al., 2019). Using an additional systems biology approach, integrating transcriptomic and epigenetic information, cultured RA FLS cell lines have been stratified into two clusters characterized by patient-specific function of transcription factors. Among them, retinoic acid receptor alpha (RARα) regulated distinct functions in the TGFβ pathway in the two clusters. Unexpectedly, RARα depletion increased proliferation and invasion of FLS derived from one group of patients but had little or even the opposite effect in FLS from the other group (Ainsworth et al., 2022). The future use of such an approach to FLS or other cells across the RA trajectory could provide additional insights into mechanisms that explain the diversity of responses to targeted agents.

The use of longitudinal RNA sequencing of peripheral blood of RA patients under stable pharmacological treatment has recently informed events leading to disease flares (Orange

et al., 2020). This experimental approach has identified circulating blood CD45^{-}CD31-PDPN+ pre-inflammatory mesenchymal (PRIME) cells which harbor a signature of synovial sublining FLS (*FAP*, *DKK3*, *CDH11*) and seem to be recruited one week before flare. This fascinating model is consistent with the finding that synovial FLS might traffic towards cartilage implants and could passively transfer synovial inflammation in mice (LeFevre et al., 2009). Recently, high definition molecular and histological profiling of synovial tissue collected from a biopsy-driven, randomized clinical trial in RA (R4RA) (Rivellese et al., 2022) failed in its primary endpoint, namely that B cell depletion would be most effective for patients with high B cell infiltration into the synovium. However, a secondary analysis suggests an association with response to the IL-6R inhibitor tocilizumab in RA patients whose synovium was enriched for the fibroblast markers FAP and DKK3^{-} FLS of this phenotype have aggressive profile in cancer environment (Ferrari et al., 2019).

Finally, analysis of the FLS-glycome has recently been performed (Wang et al., 2021). In healthy conditions, FLS show higher amounts of a 2–6 sialylation that prevents inflammatory actions of galectins, which are a family of proteins that bind to galactose-containing glycans modulating synovial inflammation (Pearson et al., 2018; Filer et al., 2009; Forsman et al., 2011). During inflammation, sub-lining THY1+ FLS become hypo-sialylated allowing galectin-3 to bind to glycoconjugates located on the FLS surface. This promotes release of pro-inflammatory mediators (e.g., IL-6, TNF) amplifying an autocrine pathological loop. A significant increase of a2-6 sialic acid expression was found in the synovial membrane of RA patients in sustained clinical and ultrasound remission compared to OA and naive to treatment RA patients (Wang et al., 2021).

EPIGENETIC MODIFICATIONS TO STROMAL CELLS

More than 2 decades ago, it was demonstrated that FLS isolated from synovial tissue of RA patients with end-stage disease are characterized by an aggressive and invasive phenotype that is maintained even after engraftment into SCID mice (Muller-Ladner et al., 1996). This suggests that synovial FLS undergo epigenetic or genetic modifications that might be responsible for the cellular priming toward pathogenic phenotype (Ai et al., 2015). In RA FLS, early studies have linked their invasive and aggressive phenotype with hypomethylation causing an increase of expression of genes related to inflammation and proliferation (Karouzakis et al., 2009), as well as genes promoting their destructive phenotype (Karouzakis et al., 2011), though subsequent studies using whole genome bisulfite sequencing has shown similar levels of methylation albeit differential distributed throughout the genome (Ai et al., 2018). The study of global hyper- and hypo-methylation status might have limitations since individual disease-related genes might be hypo-methylated to be hyper-expressed while other disease-repressing genes must be hyper-methylated to be repressed. Recently, a cross-sectional analysis of the methylome of RA synovial tissue obtained at different disease stages has shown that these changes occur very early during the disease trajectory and significantly increase overtime directly with the disease progression. In particular, the total number of hypermethylated GpG islands was found to increase from 9% at the stage of undifferentiated arthritis up to 96% in RA patients with disease duration >3 months (Karouzakis et al., 2018). The potential for remodeling the epigenetic profile as a therapeutic modality has been suggested by studies with DNA methyl transferase inhibitors, which decrease DNA methylation and suppress severity of a murine arthritis model (Petralia et al., 2019).

Prolonged exposure to TNF remodels chromatin accessibility in synovial FLS through hyperacetylation of histones (Sohn et al., 2015; Loh et al., 2019). These modifications prime FLS toward a pro-inflammatory program and increase production of mediators like IL-6, CXCL9 and CXCL11. The potential for epigenetic remodeling as a therapeutic intervention has been confirmed by the observation that decreasing histone acetylation through histone deacetylase inhibitors represses experimental arthritis in mice (Lin et al., 2007; Cantley et al., 2015; Sohn et al., 2015). Histone modifications can also be modified by targeting histone acetylation reader proteins as BET bromodomain proteins, which repress the aberrantly activated FLS status. A BET-inhibitor (I-BET151) reduced FLS proliferation as well as their release of pro-inflammatory and destructive mediators, after *in vitro* stimulation with TNF or IL-1β (Klein et al., 2016). Another BET inhibitor (JQ1) has shown a similar beneficial effect not only *in vitro*, decreasing FLS proliferation and their release of pro-inflammatory molecules, but even *in vivo* protecting mice from collagen-induced arthritis (Xiao et al., 2016).

Recently, the epigenetic landscape of synovial FLS in RA has been mapped by integrating the whole transcriptome, DNA methylation, chromatin accessibility, and histone marks across the genome. Using a whole genome bisulfite sequencing, "differential" methylation as opposed to global hyper and hypo methylation in RA FLS was confirmed (Ai et al., 2018). RA FLS are imprinted and biased toward activation of the inflammatory transcriptional machinery. Differentially modified regions from these datasets can be mined for potential pathogenic pathways and therapeutic targets. For example, the Huntington's disease pathway unexpectedly emerged as a prominent feature of RA FLS. One protein in this pathway, namely Huntingtin-interacting protein-1 (HIP-1) regulates a variety of FLS functions. HIP-1 has been shown to be crucial in regulating cell movement in cancer (Li et al., 2017; Wang et al., 2017) and of interest, HIP-1 deficiency significantly decreased arthritis severity in a mouse model (Laragione et al., 2018). Some of the pathways identified to be under epigenetic control in RA FLS (Laragione et al., 2018) were found to be shared in CD3^{+} T cells as shown by the paired analysis of synovial tissue and peripheral blood samples from RA patients (Ai et al., 2021). Therefore, extensive understanding of the epigenetic patterns regulating immune and stromal cell behavior not only in early or established RA but even in the pre-clinical phase has the potential to identify additional diagnostic, prognostic, and therapeutic targets.

Remodeling the imprinted state of FLS can also be achieved in other ways. For instance, a recent phase 1b/2a clinical trial using a cyclin-dependent kinase inhibitor (namely Seliciclib) suppressing the expression of survival protein blc2 has been conducted (Pratt et al., 2021); however, the clinical efficacy of Seliciclib in RA has not yet been reported. FLS were also targeted in RA clinical trials with an anti-cadherin 11 antibody, although efficacy was observed (Finch et al., 2018). As more data accumulate

defining synovial fibroblast phenotypes and functions, it is likely that unique mechanisms will be identified that can be targeted to return FLS to their homeostatic state.

MYELOID CELLS PERPETUATE AND REGULATE SYNOVIAL INFLAMMATION

Studies in animal model of arthritis and in rheumatoid synovium, the impact of myeloid derived cytokines (e.g., TNF, GM-CSF, IL-6, and multiple chemokines) have placed synovial macrophages as a critical component of most pathogenetic models. Recently, an integrated single taxonomy for synovial tissue macrophages (STM) has been proposed (Kurowska-Stolarska and Alivernini, 2022). In human synovial tissue, there are two main populations of macrophages identified based on their expression of MerTK, which is a tyrosine kinase receptor and CD206, that is a C-type lectin mannose receptor. These populations contain multiple distinct clusters whose enrichment dynamically changes across different stages of RA progression (Alivernini et al., 2020) (Figure 2). In synovium from healthy humans, resident MerTK+CD206+ synovial tissue macrophages are the predominant subtype (Alivernini et al., 2020; Culemann et al., 2019). Among them, the TREM2+CX3CR1+FOLR2+ cluster forms a protective lining layer in the synovial membrane, while LYVE1+FOLR2hi cluster mostly resides in the synovial sublining layer (reviewed in Kurowska-Stolarska and Alivernini, 2022).

Immunohistochemical analysis of the Pathobiology Early Arthritis Cohort (PEAC) has revealed different pathotypes of synovial tissue inflammation: (1) diffuse myeloid, characterized by monocyte or macrophage enrichment, (2) lympho-myeloid showing aggregates of B and T lymphocytes, and (3) fibroid, which has only limited inflammatory cell infiltrate (Humby et al., 2019). More recently, multimodal single-cell integration analysis reveals a more complex heterogeneity of synovial inflammation with up to six distinct cell type abundance phenotypes (CTAPs) from a prominent T and B cells enrichment (CTAP-TB) to a more balanced combination of myeloid, fibroblast, and endothelial cells mostly lacking lymphocytes (CTAP-EFM) (Zhang et al., 2022). To date, correlations between the pattern and clinical phenotype or response to therapeutic agents have not been confirmed. In at least two of the most common types of synovitis, inflammatory macrophages (MerTK-CD206-) are key cellular components. In the synovium of naive-to-treatment or resistant-to-cDMARD RA patients, the CD48+S100A12+ cluster is the most abundant. They express alarmins (S100A8, S100A9, and S100A12) and CXCL8, which confer the ability to activate monocytes and FLS and to promote chemiotaxis of neutrophils. A CD48+SPP1+ cluster expressed osteopontin suggesting bone damage potential and glycolytic enzymes, cytoskeletal proteins and integrins suggesting migratory properties (Alivernini et al., 2020).

Recently, signaling lymphocytic activation molecule family member 7 (SLAMF7 or CD319) emerged as a receptor implicated in the induction of an activated status of macrophages in chronic inflammatory disorders, including RA (Simmons et al., 2022). Whereas SLAMF7 was expressed at low amounts in synovial tissue-derived macrophages from OA patients, expression was 40-fold higher in RA STM. An STM SLAMF7 stimulation signature drives a dominant myeloid inflammatory program characterized by up-regulation of chemokines as well as inflammatory cytokines representing a detrimental autocrine loop. The synovial MerTK-SPP1+ myeloid cluster was enriched for SLAMF7 supporting their pathological role. Finally, the transcriptomic signature of a CD48+ISG15+ cluster suggests a population enriched for type I IFNs and high amounts of APRIL, which could support the formation of synovial ectopic germinal centers (Alivernini et al., 2020; Humby et al., 2019). Activation of this cluster is likely driven by microRNA-155, which is a potent post-translational regulator of inhibitors of the inflammatory response (SHIP1 and SOCS1) (Kurowska-Stolarska et al., 2011) in treatment-resistant RA, promoting polyfunctional $CD4^+$ T-cell activation (Olsson et al., 2022) and resistance to apoptosis (Rajasekhar et al., 2017). Single-cell RNA sequencing analysis of paired peripheral blood and synovial tissue samples of ACPA− compared to ACPA+ patients of variable disease duration (ranging from months to >10 years) found no significant difference in terms of peripheral blood-derived monocytes, whereas ACPA- RA patients synovium expressed enrichment of HL-DRB5-CCL+ and IL18+CCL+ macrophages (Wu et al., 2021). These are characterized by increased expression of *CCL13*, *CCL18*, and *MMP3*, suggesting the existence of distinct cellular pathways in the pathogenesis of seropositive and seronegative RA.

RA patients in imaging remission show histological signs of residual synovitis in terms of persistent sublining macrophage and T lymphocyte infiltration when compared to ''naive-to-treatment'' active RA (Alivernini et al., 2017, 2021). Remission synovium is characterized by a distinctive STM appearance (Alivernini et al., 2020) with predominance of inflammation-resolving MerTK+CD206+ and inflammatory MerTK- CD206- STMs. Synovial tissue of RA patients in sustained Boolean-defined remission is enriched for MerTK+CD206+ STMs (Alivernini et al., 2020). RA patients in sustained disease remission might experience disease flare immediately after treatment cessation and approximately 50% will flare within a year (Alivernini et al., 2016). The ratio between inflammation-resolving MerTK+CD206+ and pro-inflammatory MerTK-CD206- STMs predicts persistent remission when therapeutics are tapered and discontinued in RA patients (Alivernini et al., 2020). This concept is supported by the limited duration of drug-free remission (Ajeganova and Huizinga, 2017) with progressive risk in disease flare over time (Heimans et al., 2016), clearly suggesting that drug-free remission in RA is fragile and is not equivalent to the self-sustained homeostasis of healthy joints. Establishing the function of these gene pathways in the synovium will provide new insights about the mechanisms that prime the tissue as a ''chronically inflamed'' niche increasing the chance of remission loss.

Although the mechanisms that govern the super-repressed gene expression pattern in remission are unknown, possible candidates include the action of autoantibodies and/or epigenetic factors. Autoantibody (ACPA and RF) positivity is related to a higher probability of disease flare after treatment modification (Haschka et al., 2016; Rech et al., 2016); reversal of ACPA positivity is an uncommon phenomenon in RA patients regardless of sustained disease remission (Boeters et al., 2019). This supports the concept that persistent autoantibody positivity may be responsible for triggering new onset of inflammation leading to disease flare. An alternative consideration is that MerTK+CD206+ STMs and their local precursors bear

epigenetic imprinting linked to previous inflammation. Fate-mapping studies conducted on mouse models suggest that murine counterparts of TREM2+ and FOLR2+LYVE1+ STMs can be long-lived (Misharin et al., 2014; Culemann et al., 2019), and this can increase their susceptibility to epigenetic imprinting within the synovial environment. It is possible that a persistent decrease in expression of tissue immune-tolerance mechanisms of healthy synovial tissue characterizes a previously primed biological niche in a "chronic inflammatory" trait that might lead to recurrence of inflammation in disease flare.

The development of *in vitro* micro-coculture systems permits evaluation at single-cell resolution, of the interaction between synovial stromal and immune cells. Coculture of FLS with proinflammatory MerTK-CD206-, but not with MerTK+CD206+ synovial tissue macrophages, generated a distinct FLS cluster, not present in resting conditions, characterized by high expression of cartilage- and bone-destructive mediators, proinflammatory cytokines, and chemokines. Conversely, MerTK+CD206+ STM isolated from biopsies of RA patients in sustained remission-induced FLS repair responses (Alivernini et al., 2020). These findings suggest modulation of synovitis by discrete STM populations that actively impact on the stromal resident compartment in RA.

DO T CELLS DRIVE RA CHRONICITY?

It has long been established that T cells isolated from synovium of established RA patients are not a rich source of cytokines suggesting a limited contribution in the established phase of disease (Firestein and Zvaifler 2002). That said, abatacept is efficacious in established RA via CD28 pathway modulation. A multi-omic analysis of synovial tissue with dense leukocyte infiltrates from seropositive RA patients with active disease has revealed a heterogeneous resident $CD4^+$ T cell population with distinct expression patterns of five activation markers (PD1, MHC-II, ICOS, CD69, and CD38) (Rao et al., 2017). In particular, expansion of a population of "peripheral helper" PD-1hiCXCR5-$CD4^+$ T cells, characterized by the expression of genes involved in B cell cross-talk and activation (including IL21, ICOS, CXCL13, and MAF), has been detected in synovial compartments (both tissue and fluid) of patients with RA. Similar to follicular helper PD-1hiCXCR5+ T cells, which are responsible for plasma cell differentiation by IL12 and SMAF5 engagement (Crotty, 2011; Cannons et al., 2010), their transcriptomic signature suggested their ability to support B cell activation and autoantibody production.

Single-cell RNA sequencing and mass cytometry comparison of synovial tissue of active RA compared to OA patients (Zhang et al., 2019) identified three $CD4^+$ and three $CD8^+$ T cell subsets associated with RA. Synovial $CD4^+$ T cells are clustered as follows: CD4+CCR7+ T cells enriched of central memory immunologic gene set (*SELL*, *CCR7*, *NFKBIZ*, *LEF1*); CD4+FOXP3+ Treg cells (*FOXP3*, *CTLA4*, *TIGIT*, *DUSP4*); CD4+CXCL13+ follicular helper T cells (*CXCL13*, *CD200*, *PDCD1*). Interestingly, RA synovium with "leukocyte-rich" inflammation was characterized by the expansion of CXCL13+$CD4^+$ cluster (PD1+CD4+ICOS+ cells) compared to RA synovial tissue with "leukocyte-poor" inflammation or OA synovial tissue. The analysis of the $CD8^+$ T cell synovial compartment have revealed three clusters based on the expression of effector molecules: CD8+GZMK+ (*GZMK*), GNLY + GZMB+ cytotoxic T cells (*GZMB*, *GNLY*, *ZNF683*) and GZMK + GZMB+ effector T cells (*IFNG*, *HLADRB1*, *HLA-DPA1*), respectively, the latter being one of the major sources of synovial IFNγ release. Similarly, RA synovium with "leukocyte-rich" inflammation has been characterized by the expansion of PD1+CD8+ICOS+ cluster compared to "leukocyte-poor" tissues or OA-derived synovial tissue (Figure 2). Detailed functional evaluation of these clusters is now required.

Immunohistochemical analysis of synovial biopsies obtained from RA in sustained clinical and ultrasound (Power Doppler negative) remission has shown the variable infiltration with sublining $CD3^+$ T cells aggregates, suggesting a putative role as drivers of synovitis chronicity as well as disease flare (Alivernini et al., 2017). Late-stage non-inflamed RA synovial tissue is enriched for oligoclonal $CD8^+$ resident memory T cells (T_{RM}) resembling $CD8^+$ cells bearing T_{RM} markers found in multiple mouse models, which persist during remission in previously inflamed joints. This subset can drive CCL5-mediated recruitment when activated by antigen (Chang et al., 2021). In human inflamed synovial tissue, an unbiased high-dimensional single-cell RNA sequencing and FACS analysis has revealed that a $CD8^+$ T cell population expressing granzyme K (GzmK) is clonally expanded and is a major source of cytokine production fueling inflammation in both antigen-dependent and independent manner (Jonsson et al., 2022). The phenotypic characterization of residual infiltrating synovial T cells in RA in remission will be crucial to confirm their pathogenetic actions in promoting remission loss.

METABOLISM REPROGRAMMING IN RA

Metabolic deregulation is now recognized as an additional pathogenetic factor in RA (Weyand and Goronzy, 2017). In normal synovial tissue, glycolysis is the primary pathway by which mitochondrial substrate is promoted. In RA synovial cells, there is a progressive increase of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH) leading to increase of lactate concentrations in parallel with reduction of glucose concentrations (Henderson B et al., 1979). During RA progression, tissue resident, and invading macrophages, T and B lymphocytes and stromal cells are under metabolic stress. Synovial tissue inflammation creates a hypoxic microenvironment poor in glucose and rich in lactate that leads exerts significant immunomodulatory impact. For example, T cells can sense lactate and uptake by $CD4^+$ T cells inhibits migration, promoting maintenance within the inflamed synovium. It may also promote differentiation toward a Th17 cell phenotype (Pucino et al., 2017; Certo et al., 2020). Furthermore, tissue lactate influences T cell motility through SLC5A12 and SLC16A1 transporters (Haas et al., 2015)—RA synovial fluid is enriched for lactate dehydrogenase isoenzymes compared to OA (Pejovic et al., 1992). In health, LDH-A regulates T cell differentiation, promoting Th1 cell polarization and IFNγ production (Peng et al., 2016). LDH inhibition reduces $CD8^+$ T cells lipogenesis, migration, and proliferation as well as crosstalk with B cells (Souto-Carneiro et al., 2020) suggesting that a lactate rich environment might promote the creation and maintenance of lymphoid-like structures within the synovial membrane. Alterations of glucose metabolism have been detected even in FLS and can promote pathogenetic behavior. In particular, hexokinase 2, the principal inducible

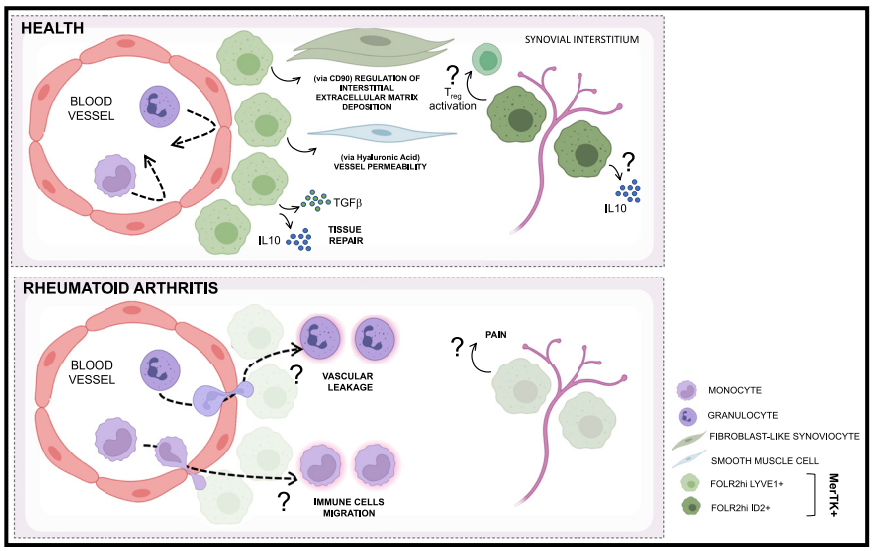

Figure 3. Schematic of putative roles of sublining interstitial macrophages in health and rheumatoid arthritis synovial tissue

The healthy interstitium contains at least two distinct macrophage clusters: perivascular and nerve-associated macrophages (NAM), across different tissues. Perivascular, FOLR2+LYVE1+ macrophage cluster produce IL-10 and TGFβ family members regulating tissue repair. Moreover, FOLR2+LYVE1+ macrophages might interact directly with $CD90^{+}$ FLS regulating interstitial extracellular matrix deposition and, via hyaluronic acid, with smooth muscle cells regulating blood vessel stiffness and permeability. NAM are located adjacent to sympathetic nerves and exert immunoregulatory functions expressing IL-10 and being potent inducers of T-reg. To date, NAM have not been formally identified in the human or mouse synovial tissue although their transcriptomic profile suggests that they might be embedded within the recently described tissue-resident LYVE1lold2+ cluster (Alivernini et al., 2020). In Rheumatoid Arthritis the reduction of FOLR2+LYVE1+ and LYVE1lold2+ synovial tissue macrophages might be responsible for increased vascular permeability and immune cells migration and well as pain.

form that catalyzes the first step in glucose metabolism, is specifically expressed in RA synovial lining layer and regulates pro-destructive FLS behavior (Bustamante et al., 2018).

Multiple metabolomic studies have been conducted seeking specific metabolic signatures in plasma or synovium with prognostic or theragnostic utility. For example, peripheral blood of RA patients is enriched for 3-hydroxybutyrate and phenylalanine concentrations which predict tocilizumab response (Murillo-Saich et al., 2021). Similarly, seven polar metabolites (phenylalanine, 2-hydroxyvalerate, succinate, choline, glycine, acetoacetate, and tyrosine) significantly discriminate responders and non-responders RA patients at baseline receiving Rituximab (Sweeney et al., 2016). Interestingly, baseline urine metabolic profiles have discriminated between RA patients who have a good response to anti-TNF therapy (Kapoor et al., 2013) suggesting that the study of metabolomic profile might help the development of novel approach for treatment stratification. All such studies however are beset by limited validation thus far.

IMMUNE NEURAL INTERACTIONS MODULATING RA INFLAMMATION

It has long been recognized that neurologic pathways can regulate peripheral inflammatory lesions. The central nervous system receives input from sites of peripheral inflammation and can, in turn, modulate the inflammatory process. More than two decades ago experiments with vagus nerve stimulation demonstrated an anti-inflammatory action within the so called "cholinergic anti-inflammatory pathway" (Tracey 2007; Borovikova et al., 2000). Peripheral axon terminals of nociceptors are located in proximity to immune cells on which they have been shown to exert modulatory effect through the alpha7 nicotinic acetylcholine receptor (α7nAChR) (Wang et al., 2003; Olofsson et al., 2012; Pavlov and Tracey, 2017). The activation of spinal cord adenosine receptor significantly represses inflammatory arthritis and joint destruction in mouse model. Various immune cells as macrophages express α7nAChR (De Jonge and Ulloa, 2007) in which its agonists suppress the production of pro-inflammatory cytokines (i.e., IL-1, TNFα, and IL-6) after LPS stimulation (Wang et al., 2003; Borovikova et al., 2000). RA synovium has been shown to express α7nAChR as well as cultured FLS in which a significant reduction of pro-inflammatory cytokines has been seen after α7nAChR stimulation (Waldburger et al., 2008). Vagal stimulation induces the release of acetylcholine from splenic choline acetyltransferase-positive T cells (Rosas-Ballina et al., 2011), which inhibit the nuclear translocation of NF-KB in macrophages through α7nAChR (Lu et al., 2014a; Rosas-Ballina et al., 2009).

An attempt to test the immunomodulatory effect of vagus nerve electric stimulation *in vivo* in RA patients suggests that it might repress TNF release from peripheral blood-derived mononuclear cells as well as improving disease activity. This anti-inflammatory effect was seen in early RA patients not responding to csDMARDs as well as in a cohort of RA patients with difficult to treat disease (Koopman et al., 2016). Controlled clinical trials are needed to determine the clinical utility of this approach.

In the sublining layer of the human synovial tissue, sympathetic and sensory nerves are also present, providing a link between the synovium and systemic responses to changes within the tissue environment. The healthy interstitium across several tissues (e.g., skin, lung, heart, and fat), contains two distinct macrophage clusters with different homeostatic functions related with their tissue location as nerve-associated macrophages (NAMs) and perivascular macrophages (Chakarov et al., 2019). NAMs are in proximity of the sympathetic nerves and are tissue-resident macrophages with low LYVE1 and high MHCII expression, respectively. Multiple evidence in animals suggests that NAMs and nerve interaction is of crucial importance for the regulation of the inflammatory cascade (Chakarov et al., 2019; Ural et al., 2020). In particular, NAMs have an immunoregulatory phenotype, via release of IL-10 (Ural et al., 2020). NAMs can provide essential cues for nerve homeostasis, suggesting two-way communication between macrophages and the adjacent nerves. Depleting NAMs in the gut alters the normal

peristaltic activity since NAMs are a source of bone morphogenic protein 2 (BMP2) that binds to its receptor (BMP2R) on enteric neurons promoting gut neuron-mediated motility (Muller et al., 2014). To date, NAMs have not been characterized in the synovial tissue, although their transcriptomic profile suggests that they might be embedded within the recently described tissue-resident LYVE1loId2+ STM cluster (Kurowska-Stolarska and Alivernini, 2022). Their comprehensive characterization on synovial tissue samples obtained from at-risk individuals and from RA patients across disease phases will be crucial to improve our understanding on the causative mechanisms of pain in the preclinical stages as well as the etiology of sustained pain that afflicts patients with active RA and that might persist despite inflammation resolution (Figure 3).

CONCLUDING REMARKS

Our current understanding of the complex immune pathogenesis of RA has been summarized in this review. Appropriate application of the range of novel immune analysis modalities now available will continue to deconstruct the complex pathogenetic network that drives this systemic autoimmune disease. In particular, the spatial distribution of distinct cells in tissues generally mirrors the functional diversity among cells and the differences in their fate and lineage. Therefore, the integration of spatial datasets, single-cell sequencing and sequential polyomic data integration and systems analysis over different disease stages, will provide the opportunity to improve our understanding about the composition and function of tissues over time. This improved systematic understanding of pathogenesis will hopefully offer precision therapeutics delivered with the promise of improved safety and outcomes.

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