Review

One year in review 2020: pathogenesis of rheumatoid arthritis

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ABSTRACT
Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease influenced by both genetic, epigenetic and environmental factors. The discovery of new gene polymorphisms and their association with disease susceptibility have added new elements to better clarify RA pathogenesis. In the last year, important elements have been added to the current knowledge of mechanisms regulating innate and adaptive immunity in RA, leading to discovering new targets for the development of disease-modifying therapies. Thus, in this review we summarise the new insights resulting from a literature research of the data published in the last year.

Introduction
Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease influenced by both genetic, epigenetic and environmental factors. Over the last few years, particular attention has been given to novel genes polymorphisms associated with disease susceptibility and with different stages of the disease (1). Several elements of the environment and particular lifestyles such as diet and physical exercise seem to influence the development of the disease and some of its clinical aspects. The cellular elements and the soluble components of the innate and adaptive immune system exert direct and/or indirect effects on tissue inflammation and remodelling both systemically and locally. In light of the new research novelties, both innate and adaptive immune systems are dynamic and play active roles in RA pathogenesis. The most recent findings concerning RA pathogenesis have been systematically described during the last years (1-5). In the current review we reported a Medline search of articles in English published in the PubMed database from 1st January 2019 to 31st December 2019.

Genetic aspects
Genome-wide association studies (GWAS) and subsequent meta-analyses were able to identify different alleles that govern RA susceptibility, especially the major histocompatibility complex (MHC) genetic variants. Beyond disease susceptibility, it has been recently suggested that distinct HLA alleles may define different serological phenotypes of the disease. Anti-citrullinated peptide antibodies (ACPA), assayed with the commercial CCP2 assay, are highly specific to RA. Recently, Terao et al. explored how individual serologies linked to RA drive associations within the MHC. They were able to find a cluster of tightly co-occurring antibodies (canonical serologies, containing CCP2), along with several independently expressed antibodies (non-canonical serologies). Interestingly, unique genetic characteristics underlying fine-specific ACPA have been suggested after having demonstrated that in CCP2- RA an association between CCP2 - RA and B^{*}9 was derived from individuals who were positive for non-canonical serologies. Similarly, in CCP2+ RA the associations between subsets of CCP2+ RA and B^{*}9 were negatively correlated with the number of positive canonical serologies (6), suggesting that RA may be further subdivided beyond simply seropositive and seronegative. Recently, besides the HLA loci, several genetic variants in non-HLA loci have been associated with the disease. For example, a number of single nucleotide polymorphisms (SNPs) may modulate the production of several cytokines involved in RA development and progression. In this context, the INFG-1616 G and INFG-1616 GG genotypes have been associated with disease susceptibility (7) and TNF-α -308A with both susceptibility and severity of the disease (8). Among the different cytokines regulating RA

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pathogenesis, IL-12 seems to modulate processes involved in the immune response and self-tolerance. In particular, the rs17860508 polymorphism of IL12B gene was recently associated with genetic susceptibility to RA in the Bulgarian population (9). Furthermore, a number of genetic variants may contribute to disease susceptibility by affecting the activity of RA synovial fibroblasts (FLS), key cells responsible of tissue remodelling and cartilage damage in the joint of RA patients. For instance, the rs26232 C allele, located in intron one of C5orf30, associated with the susceptibility to and severity of RA, was recently proved to be also associated with RA FLS with more profibrogenic activities and with increased expression of the adhesion molecules ICAM-1 and IP-10 (10). Moreover, different gene variants seem to be associated with specific clinical aspects of the disease, rather than with disease susceptibility. This is the case of RARB gene (rs1616199914) linked to the development of subclinical atherosclerosis in RA. This data is extremely important given that cardiovascular disease is the most common cause of morbidity and mortality in RA (11). Other different genetic variants have been associated with disease susceptibility, including SNPs of the Angiopoietin-2 (Ang2) gene (12), the T/T genotype for rs3738581 SNP of the Semaphorin 4 A (sema4A) gene (13) and the newly discovered non sense SNP in SUPT20H gene (SUPT20H:c.73A>T (p.Lys25*)) (14). However, further investigations are required to clarify the precise role of these genetic variants in different processes of RA pathogenesis.

Growing interest has recently been given to a number of loci which may be associated with susceptibility to different autoimmune diseases. Among these shared risk loci, one of the most studied is the TNF-α-induced protein 3 (TNFAIP3) gene, encoding for the A20 protein, which is a negative regulator of NF-kB. Deficiency of this gene in immune cells has been linked to inflammation and autoimmunity. The rs6920220 SNP in TNFAIP3 gene was found to be significantly associated with susceptibility to RA as well as systemic lupus erythematosus (SLE) and primary Sjögren syndrome (pSS) in an Italian population (15). In parallel, the missense variant (1858C>T) of the protein tyrosine phosphatase non-receptor 22 (PTPN22) gene, encoding the lymphoid protein tyrosine phosphatase LYP, a negative regulator of T and B cells activation, has been associated with over 20 different autoimmune diseases, including RA. Ruiz-Noa et al. have recently demonstrated that PTPN22 1858C>T polymorphism is associated with increased CD154 expression and higher CD4+ T cells percentage in RA patients, contributing to the inflammatory processes of the disease (16). Although different genetic variant in HLA and non-HLA loci are thought to be crucial in RA pathogenesis, they cannot explain disease susceptibility alone. An increasing body of evidences support the importance of epigenetics in RA pathogenesis. Epigenetic modifications are inheritable and potentially reversible changes in DNA and chromatin that regulate gene expression without altering the DNA sequence. The major epigenetic mechanisms include DNA methylation, histone proteins modifications and changes in gene expression by microRNA (miRNA) and other non-coding RNA. In particular, miRNA are small non-coding RNA molecules that are implicated in post-transcriptional gene expression regulation. Thus, miRNA are able to negatively regulate gene expression by binding the 3’-untranslated region (3’-UTR) of their target messenger RNA (mRNA), resulting in inhibition of their translation and down-regulation of corresponding proteins. Therefore, particular interest has been given to investigate the association between altered miRNA levels with disease susceptibility or activity in RA. For example, a recent analysis of miRNA expression profiles demonstrated that miR-5571-3p and miR-135b-5p expression levels were positively correlated with inflammation and disease activity (17). Subsequently, other miRNA were investigated and related to different aspects of the disease. In this context, Romo-García et al. identified a distinctive set of 97 miRNA that are over-expressed in early RA, including miR-23a-3p, miR4634 and miR 361-5p, not yet described in this autoimmune disease. The putative target genes of these miRNA are significantly enriched in inflammation, apoptosis and tolerance loss pathways (18). Notably, a target gene related to tolerance loss, according to the gene ontology analysis, was RUNX3, able to modulate inflammatory processes by directly interacting with Foxp3 protein and to promote T cells development. On the other hand, some miRNA can also affect the processes of bone erosion and joint destruction by regulating the levels of metalloproteinases (MMPs), active regulators of tissue remodelling. For instance, it has been recently reported that the over-expression of miR-145-5p in RA FLS increased MMP-9 production by NF-kB pathway, thus contributing to accelerate bone erosion (19). However, some miRNA may also play protective role in RA by down-regulating the expression of proteins that are involved in inflammation and/or joint destruction. For example, miR-613 may contribute to suppress proliferation and invasion of RA FLS by directly down-regulating the expression of DKK1, a Wingless (Wnt) signalling pathway inhibitor that was considered as a master regulator of bone destruction and joint remodelling. The protective role of miR-613 could be reduced in RA patients, since miR-613 expression was found to be significantly down-regulated in RA synovial tissue and FLS (20). As miR-613, the newly discovered miR-22 might exert a protective role in RA by inhibiting in RA FLS proliferative and pro-inflammatory activities and by targeting SIRT1 (21). In support to the role of miR-22 in RA, this resulted down-regulated in the synovia of RA patients as well as miR-27b-3p in the cartilage tissue (22). Beyond the pathogenic role of miRNA in RA, different epigenetic mechanisms are currently under investigation, including DNA methylation changes. A recent sequencing-based high-resolution methylome mapping revealed biologically relevant DNA methylation changes in asymptomatic individuals positive for ACPA, which can be detected even prior to clinical manifestations as evidence of early immune dysregulation. Furthermore, the gene associated with these differentially methylated regions were...
involved in immune-related pathways (including CARD11, CSF2, MAP3K7, NFATC1, PAK4, NFKB1A, MAPK9, IFNAR2, FCGR2A and SOCS3), as well as in several viral infections (i.e. EBV) related pathways (23). Consistently with the well-known role of EBV in triggering RA, these results suggest that altered DNA methylation prior the disease onset may be particularly relevant for environmental triggers. The role of DNA methylation in the pathogenesis of RA has been widely evaluated in past years. In order to provide a comprehensive, accurate and reproducible analysis of DNA methylation, the newer whole-genome bisulphite sequencing method (WGBS) has been recently developed. While performing the first investigation of whole-genome DNA methylation of FLS from RA and osteoarthritis (OA) patients through WGBS technology, Ham et al. identified 523 methylated regions (LMRs) specific to RA. Furthermore, some RA-specific LMRs overlapped with specific motifs that are closely related to TGFβ pathway, such as GLI1, RUNX2 and TFAP2A/C whose role in RA pathogenesis has been previously reported (24). Histone methylation has been another hot topic in the field of epigenetics, since this histone modification can affect the interaction between DNA and histones, leading to structural changes in chromatin. Abnormal methylation levels of H3K27 and H3K4 in thymic Zbtb16 (encoding PLZF) and Tbx 21 (encoding T-bet) promoter regions were associated with developmental and differentiation defects in invariant natural killer T (iNKT) cells in DBA/1 mouse RA model in which the transcriptional killer T (iNKT) cells in DBA/1 mouse differentiation defects in invariant natural killer T (iNKT) cells in DBA/1 mouse Zbtb16 (encoding PLZF) and Tbx 21 (encoding T-bet) promoter regions were associated with developmental and differentiation defects in invariant natural killer T (iNKT) cells in DBA/1 mouse (25).

Take home messages
- SNPs in several cytokine genes have been associated with RA (7, 8, 9).
- RARB gene (rs1161199914) is linked to the development of subclinical atherosclerosis in RA (11).
- Altered miRNA levels are associated with disease susceptibility and activity in RA (17).

### Genetic features

| Genomic position | SNPs (rs2069705) | TNF-α -308A genotype | IL12B (rs17860508) | r26232 C allele | RARB (rs1161199914) | SNPs of Ang2 | SEMA4A (rs3738581) | SUPT20H: 73A>T (p.Lys25*) | TNFAIP3 (rs6920220) | IL5R (rs4634) | miR-135b-5p | miR-23a-3p | miR-145-5p | miR-361-5p | miR-145-5p | miR-5571-3p | miR-122 |
|-----------------|-----------------|----------------------|-------------------|-----------------|---------------------|-------------|------------------|------------------------|---------------------|-----------------|--------|-----------|---------|----------|---------|----------|---------|---------|--------|
| Susceptibility  | Susceptibility and severity | Susceptibility | Susceptibility | Subclinical atherosclerosis | Susceptibility | Susceptibility, severity, tissue remodelling | Susceptibility | Susceptibility | Susceptibility | Susceptibility | Severity | Severity | Severity | Susceptibility | Susceptibility | Susceptibility | Susceptibility |

• Some miRNA may also play protective role in RA (20, 21).
• Altered DNA methylation may play a central role in RA pathogenesis (24).

### Environmental factors

The environment, including smoking, air pollutants, diet, obesity and infections have been proposed to trigger RA in genetically predisposed individuals. Thus, several studies were conducted in humans and animal models in order to better define these mechanisms and subsequently to identify new potential therapeutic targets.

#### Cigarette smoking

Recently, the exposure to air pollutants has been implicated in the occurrence of autoimmune diseases such as RA, providing the lung as an autoimmune initiation site. The underlying mechanisms may include systemic inflammation, increased oxidative stress and airway damage, leading to immune responses. Cigarette smoking (CS) is the main environmental factor that has been linked to an increased risk of developing RA and a more severe disease (26). A prospective study in a large cohort of female population confirmed the association between active smoking and the risk of developing the disease. This study demonstrated for the first time that passive exposure to tobacco in childhood might increase the risk of developing the disease. These evidences perfectly fit with the hypothesis that external events, occurring at an early stage of RA, might trigger autoimmune susceptibility (27). In fact, it is well known that CS is able to induce the production of rheumatoid factor (RF) and ACPA, autoantibodies linked to joint destruction and systemic bone loss even in the early phases of RA. However, this effect has been shown to be specific for ACPA production, is independent of ethnicity and results from the interaction between CS and SE alleles (28). Interestingly, aromatic hydrocarbon receptor (AHR) and its downstream gene expressions resulted highly expressed in peripheral blood mononuclear cells (PBMC) from RA smoking patients compared to non-smoking ones, suggesting that smoking may be involved in RA pathogenesis also via AHR pathways (29).

#### Air pollution and occupational and atmospheric agents

Several studies evaluated the role of particulate pollutants in the development of RA. It seems that cellular components of the airways are able to process and subsequently present air particles as antigens, triggering the activation of the adaptive immune system. Two different Swedish population-based-case-control studies discussed the possible role of occupational exposure to inorganic and organic dusts in the development of RA. Exposure to the inorganic dusts such as asbestos and silica resulted in a higher risk of developing both seropositive and seronegative RA in male workers, suggesting that inorganic dusts and CS might act differently on the induction of the disease (30). In parallel to the inorganic dusts, animal and...
textile dusts, among the organic ones, seem to be associated to the development of the disease. Furthermore, this risk increased with longer duration and/or higher intensity of exposure in seropositive RA patients. Thus, It has been hypothesised that these effects are due to the bacterial endotoxins present in the organic dusts, responsible for activation of the immune system and consequent airways inflammation (30). These data have been confirmed in a combine model of collagen-induced arthritis (CIA) and airway inflammation induced by organic dust extract (ODE). The combination of these animal models resulted in the greatest degree of arthritis and bone loss (31), suggesting the direct contribution of organic dusts to the development and perpetuation of RA.

**Lung mucosa**
The direct link between mucosal surface and RA development has been proposed. This has been recently confirmed in different case control studies in which RA was strongly associated with asthma (32). In particular, It has been observed that clinical-diagnosed asthma was associated with increased risk for ACPA production, independently of confounder elements, including smoking intensity and/or duration, suggesting that inflammation and damage of the asthmatic airways may lead to protein citrullination and other post-translational modifications (33).

**Microbiota and infections**
The association between viral and bacterial infections and RA development has been deeply investigated. It seems that both viruses and bacteria might induce citrullination processes, required for the production of ACPA. In addition to the already described bacteria such as Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans (34), several components of the gut microbiota seem to be involved in different manner. For example, the fecal Prevotella spp has been found in RA subjects at pre-clinical stages (35) and the Phylum Bacteroidetes in the early stage of the disease. Functional analysis of these bacteria, based on clusters of orthologous groups [COGs], revealed that iron transport-related genes were enriched in early RA patients. Since the iron transport-related genes are associated with membrane receptor proteins binding iron-chelating siderophores, it seems that this mechanism contributes to the development of anaemia in RA patients. Furthermore, the gene of sugar transferases involved in lipopolysaccharide (LPS) synthesis, COG2148 was enriched only in RA patients in which this was positively correlated to the disease activity score (DAS28) (36). On the other hand, analysis of RA prevalence in a historical cohort of 56,000 Danish people underwent urea breath-test (UBT) with or without H.Pylori (HP) infection, did not show any association between this bacteria and RA, not supporting the involvement of HP in RA development as previously proposed (37). New insights suggest that the intracellular bacterium Mycobacterium avium subsp. Paratuberculosis (MAP) play a role in RA as well as in other autoimmune diseases by inducing a dysregulation of macrophages, involving mechanisms such as molecular mimicry and citrullination. Bo et al. analysed the immune response against PtpA and PknG in a case-control study of RA patients compared to healthy controls. PtpA (protein tyrosine phosphatase A) and PknG (protein kinase G) are crucial for the survival of the pathogen within macrophages. They found significantly higher levels of antibodies against MAP proteins in the sera of RA but not of healthy subjects, probably due to their previous MAP exposure. Thus, MAP infection may trigger citrullination to counteract pathogen elimination from the cells. In a previous study they also identified a high sequence homology among interferon regulatory factor 5 (IRF5), EBV-antigen BOLF1, and MAP antigen MAP_4027, suggesting that IRF5 might be a potential autoimmune target in RA. Analysing the correlation of immune response to these proteins, they hypothesise that MAP infection may induce a secondary immune response through cross-reaction with IRF5, contributing to the understanding the synergy between EBV and MAP infection in genetically predisposed subjects (38). However, further studies are needed in order to better understand whether citrullination is part of a process generated by the host to eliminate MAP or if other mechanisms are involved. Despite EBV, in a large observational study including more than 24000 of incident RA, a significant correlation between viruses such as parainfluenza, coronavirus, and metapneumovirus and the occurrence of the disease has been reported, suggesting that respiratory viral infections might be novel risk factors for developing RA (39).

**Obesity and diet**
RA development and disease activity are strongly related to overweight and obesity. A recent meta-analysis confirmed an increased risk of developing RA in overweight and obese subjects compared to normal weight population. Stratified by sex, the RA risk was higher for obese women (40). A high Body Mass Index (BMI) is linked with high disease activity and disability at disease onset, being an independent factor of less response to therapy. In this setting, Aliverminini et al. matched systemic and local inflammatory environments in a cohort of RA patients in different disease phases and according to their BMI. In overweight/obese naive-RA they found an increase of follicular synovitis and sub lining inflammatory cells (CD68+, CD21+ and CD20+) compared to normal weight naïve-RA, and this trend has been confirmed at systemic levels. Interestingly, the overweight/obese naïve-RA were less responder to therapy regardless the synovitis pattern. A higher expression of sub lining CD68+, CD20+ cells and lining and sub lining CD3+ cells is also found in overweight/obese-RA in stable clinical and remission phases compared to normal weight RA. Finally, gene expression profile analysis revealed that synovial tissue of overweight/obese DMARDs naïve-RA, compared to normal weight RA, is enriched by inflammatory gene CCL3 and MyD88 (myeloid differentiation primary response gene 88), a central adaptor molecule for the majority of Toll-like receptors (41). Among the mediators involved in the adipose tissue inflammation, adiponectin is able to
stimulate RA FLS promoting Tfh cell generation, mainly by soluble IL-6, suggesting a novel role for adiponectin in RA pathogenesis (42). Beside the overweight and obesity status, the life styling seems to be relevant for prevent the development of RA. Increasing time spent in physical activity was associated with a reduced risk of the disease, independently of other risk factors including smoking, BMI history and dietary intake. Long-term physical activity had similar associations in both seropositive and seronegative patients. Liu et al. found that some of the effects of physical activity on seropositive RA was mediated by changes in BMI, suggesting that both physical activity and weight loss interventions could delay or even prevent the onset of seropositive RA (43). However, not all studies led to a common conclusion and some of them have given conflict results. This is the case of a recent study that evaluated the effect of physical exercise (PE) on the joints of adjuvant-induced arthritis (AIA) rat model demonstrated that PE was able to exacerbate joint inflammation and tissue destruction, leading to the perpetuation and exacerbation of arthritis (44).

**Take home messages**

- The risk of developing RA is associated with active smoking and might also be increased by passive exposure to tobacco (26, 27).
- Both inorganic (e.g. asbestos and silica) and organic dusts (animal and textile dusts) may be involved in the induction of RA (30).
- Inflammation and damage of the asthmatic airways may contribute to RA development (32, 33).
- MAP infection may play a role in RA by inducing a dysregulation of macrophages and triggering citrullinisation (38).
- Respiratory viral infections (e.g. parainfluenza, coronavirus, and metapneumovirus) might be novel risk factors for developing RA (39).
- Overweight/obese naïve-RA showed increased follicular synovitis and sublining inflammatory cells (CD68+, CD21+ and CD20+) (41).

**Innate immune response**

The innate immune system is deeply involved in RA pathogenesis. Recent studies on protein profiling of RA patients have allowed us to clarify some aspects of these mechanisms so far unknown. A recent case-control study showed different expression in IFNγ signalling and mediators of adipogenesis in pre-symptomatic individuals, early RA patients and control groups. In the synovial tissue, the proteins involved in cell-cell interaction, epithelial mesenchymal transition and TGF-β signalling might be useful for distinguishing pre-symptomatic from symptomatic patients (45). Defining synovial environment and its cell phenotype is a critical step to understand the mechanisms underlying the disease, necessary for further development of novel therapies. Application of transcriptomic and cellular profiling technologies may help to identify specific synovial cell populations and their different role in these processes. Among them, THY1+CD34+HLA-DR++ has been recently described as a subgroup of RA FLS expressing MHC II and producing high levels of the IL-6 (46). In parallel, RA FLS expressing Proviral-integration site for Moloney murine leukaemia virus (PIM-1) kinase has recently described in the synovia of both RA and OA patients. Following PIM-knockdown in these cells, they reduced their proliferative and migratory activities together with reduction of MMPs and IL-6 release, suggesting an active role for PIM-1 in pro-inflammatory and pro-fibrogenic functions of FLS (47). We have to take in account that FLS function may be mediated by activation of different Toll-like receptors (TLRs) by damage-associated molecular pattern (DAMPs). The neutrophil-derived lactoferrin (LTF), recently described as an endogenous ligand for TLR4 expressed on RA FLS, is able to stimulate these cells to produce and release several pro-inflammatory cytokines such as IL-6, CCL20, IL-18 (48). However, these cells are not only targets for inflammatory components, but also active players in the immune system. These cells are able to modulate phenotype and function of immune cells such as monocytes. The expression of TREM-1 (Triggering receptor expressed on myeloid cells-1), an inducible receptor expressed mainly on CD14+ cells, is up-regulated in the synovia and in the circulation of RA patients. Interestingly, monocytes co-cultured with RA FLS induced the TREM-1 expression on monocytes, through the COX-2/PGE-2/EP-4 signalling, even if other mechanisms might be involved in this process. Thus, it has been proposed that TREM-1 might be a critical link between infiltrating CD14+ cells and synovial structural cells in RA, suggesting that TREM-1 and this might be a potential therapeutic target (49). However, monocytes might be affected not only by structural cells such as FLS but also by other synovial environment components. By analysing the protein profiling of human monocytes, Rodgers et al. demonstrated that following LPS activation under hypoxia conditions these cells changed their metabolism, by increasing carnitine-dependent fatty acid oxidation and glycolysis. Thus, all these cellular changes might be responsible for increased pro-inflammatory mediators such as CCL20 and IL-1b, leading to amplification and perpetuation of tissue inflammation. However, further studies are required in order to better understand the mechanisms responsible for fatty acid metabolism-induced CCL20 release and their potential role in recruitment of Th17 cells and in osteoclasts differentiation (50). By analysing different cells and cellular mediators in the synovial fluid of RA patients, it has been showed that only the synovial fluid derived from RA has the potential to induce the differentiation of adipose-derived mesenchymal stem cells (ADSC). This seems to be due to up-regulation of COX2, IDO, IL-6, ICAM-1, VCAM-1, PD-L1 genes, through activation of NF-kB pathway by TNF and/or IL-6. However, the ADSC might play a regulatory role in immune modulation by inducing the production of CD4+Foxp3+CD25high regulatory T cells (Tregs) and by inhibiting the pro-inflammatory markers CD40 and CD80 in activated macrophages, suggesting that cells and mediators of the inflammatory environment of the synovia might affect differ-
ently the immuno-regulatory processes (51). During RA inflammation and remodelling in RA pro-angiogenic mediators are actively involved and contribute to the persistence of these processes. Recently, Pawel et al. showed that signalling of Tie2, receptor of the pro-angiogenic mediators Ang1 and Ang2, promotes macrophages-TNF-dependent activation in the synovia. The results obtained from the in-vivo experiments demonstrated that the over-expression of Tie2 is related to a more severe arthritis with higher expression of pro-inflammatory cytokines such as IL-6, IL-12B, TNF, CCL3 and with activation of bone marrow-derived macrophages, suggesting that Tie2 might be a potential therapeutic target in RA. In parallel, human monocytes purified from healthy donors differentiate to macrophages expressing Tie2 receptor when cultured with synovial fluid from RA patients but not with their sera. These intriguing results suggest that the components of the local inflammation are responsible for changing the phenotype of monocytes in pro-inflammatory cells (52).

In RA monocytes play an active role not only as pro-inflammatory cells but also for their capacity to differentiate into osteoclast precursors in the presence of RANKL and M-CSF as demonstrated in in-vitro experiments. Activation of osteoclastogenesis at the bone site in RA is well known. The mechanisms by which circulating osteoclast precursors contribute to bone remodelling are still unclear. PGC-1β (Peroxisome proliferator-activated receptor γ coactivator 1β) is induced during osteoclast differentiation. Increased nuclear accumulation of PGC-1β was observed in RA peripheral blood CD14+ monocytes, and these cells had stronger capacity to differentiate into osteoclasts than in healthy controls. PGC-1β protein expression was positively correlated with radiographic joint destruction. The inhibition of NFATc1 activation limited the effect of overexpressed PGC-1β. These results indicated that activation of the PGC-1β/NFATc1 pathway in circulating osteoclast precursors was associated with bone destruction in RA (53). Recently it has been proposed that IL-26 might regulate osteoclastogenesis in RA through increased RANK-L expression in FLS. This cytokine belongs to the Th17-cytokine family and is produced mainly by Th1, Th17 and NK cells, but also by macrophages, FLS and macrophage-like synoviocytes in RA. RA FLS incubated with IL-26 increased their expression of IL-20RA and RANKL and following IL-26RA knockdown, IL-26-induced RANKL expression was reduced. Furthermore, IL-26 promoted osteoclast differentiation from peripheral blood monocytes in the presence of low dose of RANKL, with IL-26 exerting an additive effect. Co-culture of IL-26-pretreated RA FLS with peripheral blood monocytes also increased osteoclast differentiation in the absence of addition of RANKL (54). Parallel to monocytes, macrophages are active players in different processes underlying RA pathogenesis. For example, they seem to be responsible for protein citrullination and ACPA production in secondary lymphoid organs (SLOs) and synovial ectopic lymphoid-like structure (ELSs). A recent study on SLOs from CIA mice and synovial biopsies with ELSs from RA patients, provided evidences that macrophages are able to secrete functional PAD4, following macrophage extra cellular trap formation (METosis). Furthermore, presentation of citrullinated proteins by follicular dendritic cells (FDCs) to B cells stimulated ACPA production in germinal centre reactions (GCRs) in a T cell dependent way. All together these results introduce a novel functional role for macrophages in citrullination process and inducing autoantibody production via an FDC/T-cell dependent pathway. Therefore, selective macrophage PAD-targeting approaches may represent a future therapeutic tool for down-regulating ACPA production (55). Up to now, it is well known that neutrophil extracellular traps (NETs) play active roles in the pathogenesis of RA as well as of adult-onset Still’s disease (56). NETosis is a first line defense mechanism of the immune system, represents an early step of inflammation and it is a source of citrullinated proteins in RA. These structures obtained from RA patients are able to activate both resting macrophages and polymorphonuclear cells (PMN) in both RA-patients and healthy donors, independently of immune complex formation. However, it has been proposed that NETs exert both pro- and anti-inflammatory properties depending on the target cells. Incubation of NETs with LPS-activated macrophages is able to inhibit their IL-6 secretion, on the contrary this has not been observed with PMN, suggesting different selected roles of NETs in inflammatory processes (57).

Parallel to NETs, extracellular vesicles (EV) derived from eukaryotic cells might contribute to RA pathogenesis, confirming the role of inflammatory cell-derived structures in this disease. EV are naturally occurring vesicles released from different cell types that are delimited by a lipid bilayer and are not able to replicate due to the absence of a functional nucleus (58). They are secreted, constitutively and following activation, by different cell types. Thus, their composition differs depending on their cellular origin. Besides their well-known role in intercellular communication, they can modulate, stimulate or down-regulate immune responses, depending on the original cells. Generation of platelet derived microparticles in-vitro allowed to investigate the effects of EV in cells of the innate immunity since they are similar to those identified in the circulation of RA patients. The effect of EV on the monocytes was different according to the origin of the monocytes. While on those purified from healthy subjects they induced the production of pro-inflammatory cytokines and up-regulation of CXCR1, in those obtained from patients with RA they promoted the production of IL-10 and CD36 (59).

Take home messages

- Novel subgroups of RA FLS (THY1+CD34+HLA-DR+ and RA FLS expressing PIM-1 kinase) may play a pathogenic role in the disease (46, 47).
- TREM-1expression may be a critical link between infiltrating CD14+ cells and synovial structural cells in RA (49).
• ADSC might affect differently the immuno-regulatory processes in RA (51).
• Tie2 might be a potential therapeutic target in RA because of its ability to promote macrophages-TNF-dependent activation in the synovia (52).
• PGC-1α/NFATc1 pathway and IL-26 expression have been associated with bone destruction in RA (53, 54).
• Macrophages may play a role in citrullination process and inducing autoantibody production via an FDC/T-cell dependent pathway (55).
• EV may regulate cytokines production in RA monocytes (59).

Adaptive immune response

Several studies aimed to characterise at molecular level the mechanisms regulating the activities of adaptive immune cells in the pathogenesis of RA. Recently, Oba et al. demonstrated that the IFN-γ-enhanced HLA-DR expression on Th1 cells in the site of inflammation is a crucial step for increasing circulating CD3+HLA-DR+ EV containing host cell-derived molecules. In line with previous studies reporting high frequency of circulating CD4+ T cells in RA, they found high level of CD3+CD4+ EV with a decreased level of CD3+CD8+ EV in the serum of RA patients. These intriguing results suggest that in this disease EV may be a useful tool for monitoring the activation status of total CD4+, total CD8+ and Th1/Th1c-Type T cells (60). In this context, HLA high risk haplotype DRB1*04:01 has been reported actively involved in the activation of CD4+ T cells through recognition of six aggrecan peptides in RA patients. Two aggrecan epitopes (cit-Agg225 and cit-Agg553), bound to DRβ1*0401, activate T cell responses only in their citrullinated form; other four epitopes (cit-Agg161, cit-Agg200, cit-Agg520 and cit-Agg651) are able to bind their citrullinated form; other four epitopes activate T cell responses only in their unmodified forms in a similar way. This has been confirmed by the evidence that these activated T cells were present at elevated frequencies in RA patients in comparison with HLA-DR matched healthy controls (61).

Recent studies have identified an up-regulation of the SAMD9 gene expression in PBMC from RA patients. Up to now, SMAD9 is recognised as an anti-inflammatory factor since it reduces the mRNA expression of multiple pro-inflammatory cytokines (IL-8, TNF-α, IL-4, IFNγ) in T cells. Functional studies showed also a certain control over T cell proliferation by affecting cell cycle, as demonstrated by the increase in cells in G1-phase and reduction in S-phase in SAMD9-silenced Jurkat cells. This suggests a role of SAMD9 in the regulation of nucleotide metabolism and DNA replication and recognises this factor as a potential T cell activation marker (62).

Several molecules have been identified as potentially relevant components in the function of T cells, and some of them have been linked to autoimmune diseases. In particular, genetic polymorphisms in PTPN2 and PTPN22, two proteins that control various lymphocyte responses, increased susceptibility to develop autoimmune diseases. To better understand their role in RA pathogenesis, Figueras et al. investigated the effects of PTPN2 in T cells from RA patients and found that the inhibition of STAT1 phosphorylation by PTPNs affected the expression of different STAT-regulated target genes in activated and memory CD4+ T cells. Their results modified the way particular T cell subsets sense and interpreted common cytokine cues. The results also revealed a link between PTPN2 and regulation of follicular Th (Tfh) cells, activation of T and B responses and the controlled expression of genes associated with ELS, inflammatory cytokines (IL-17A, IL-21), transcription factors (Bcl6), immune checkpoint regulators (CD274) and chemokine receptors (CXCR4, CXCR5). This may be relevant to better understanding how T cells are directed to a commitment pathway as a response to specific TCR-antigens, helping in the prediction of adaptive immunotherapy efficacy (63).

Tfh cells gained increasing interest in RA over the last years due to their role in function, differentiation and autoantibody production of B cells. An elevated number of CD4+CXCR5+PD1hi Tfh cells along with the up-regulation of Tfh transcriptional factor Bcl6, downregulation of his antagonist Blimp1, high levels of IL-21 and the positive correlation with ESR, CRP and DAS28 was recently reported in RA (64). In parallel, a reduction of CD4+CXCR5+CD127hi/CDC4+CXCR5+PD1hi ratio has been observed, indicating an imbalance between the Follicular Regulatory T cells (Tfg) and the Tfh which instead must be in balance to maintain the immune homeostasis (65). In line with these results, Zhou et al. found an increased effector memory (TEM) T cells CD4+CCR7 CD45RA- in RA patients compared to healthy controls. It has been suggested that the different expression of CCR7 in these cells is able to regulate their migration. In fact, while TEM lacking CCR7 migrate to inflamed tissues, those that express on their membrane CCR7 migrate to secondary lymphoid organs. In parallel, the numbers of circulating PD1+ICOS+TEM cells and PD1+ICOS+TCM cells were also increased in RA patients and their proportions were correlated with two relevant disease activity markers DAS28 and ESR. Among cytokines, it has been observed that high concentrations of IL-6, IL-1β and MCP-1 is suggestive of a chronic inflammatory immune status of the disease and is a mirror of a breaking of the Th1/Th2/Th17 balance in the peripheral blood of RA patients (66). Moroz et al. observed differential cytokine profiles in both serum and synovial fluid of these patients. For instance, TNFα concentration in the synovial fluid was 2.36-folder higher than in the serum while the level of IL-6 was higher by 9 times, probably because this pro-inflammatory cytokine is produced by FLS in the affected joints. In parallel, IL-4 and TRPβ1 levels were 6-fold higher in the serum than in the synovial fluid, suggesting that a Th2/Th3-mediated immune response changes into a Th1/Th17-mediated response in the inflamed joint at later stages of disease. Inflammatory processes are strictly regulated by Treg cells, attempting to counteract their inflammatory activity as proved by comparable levels of IL-10 in both serum and synovial fluid (67). Given their involvement in the control of immune responses and in the pathogenesis of RA, Treg cells and
their subsets are deeply investigated not only to define their molecular characteristics but also to identify in these cells a potential target for developing therapies that can revert the autoimmune response. Two studies described the following three different types of Treg cells CD4+FOXP3+Helios+, CD4+CD25hi and CD4+CD25+CD127dim and observed that the expression of Helios, a transcriptional factor involved in the regulation of lymphocyte development, is higher in RA patients treated with TNFα inhibitors. This observation suggests that Helios could be a marker for the identification of Treg more than FOXP3 that can be expressed by other immune subsets (68).

As far as CD25 and CD127 are concerned, numerical differences of CD4+CD25+CD127dim Treg cells and CD4+CD25hi cells have been described in RA, SLE and normal subjects, raising the hypothesis that these sub-populations of Treg cells (69).

In addition, several subsets, such as CD8+FOXP3+, CD8+CD28- or CD8+CD103+ T cells, are generally considered to be suppressive CD8+ T cells or CD8+ Tregs. Sun et al. found a way to induce and/or expand human CD8+CD103+FOXP3+ Tregs by using TGFβ1 plus RAPA in-vitro and to test their regulatory activities. This strategy induced CD8+ Tregs capable of a potent regulatory activity in-vitro and in-vivo, with a reduction of disease severity. Th17 cells levels and with induction of self-CD4+FOXP3+ Tregs and CD4+IFNγ+ in CIA murine model. These findings may suggest defects of these specialised cells, which contribute to RA pathogenesis (70). It is well known that Th17 cells play an important role in the development and progression of RA by amplifying synovial inflammation and increasing bone destruction. Several studies focused on potential promoters of Th17 polarisation and activation. For instance, Jung et al. recently confirmed the polarising effects of Sodium Chloride (NaCl) by feeding mice with a high salt diet. The animals showed a higher proportion of Th17 cells in the spleen and joints compared to control CIA mice. In addition, studies performed in-vitro observed an effect of NaCl on Th17 differentiation in a dose-dependent manner (71).

Other molecules such as ZAP70 and NMT1, involved in regulation T cell activities, have been characterised. ZAP70 is a key molecule regulating T cell activation and apoptosis and its role was investigated in an animal model of arthritis in which a partial deletion of ZAP70 (ZAP70 ±) was made. Interestingly, they observed a similar ratio of Th17 in ZAP70 ± and control mice, but with a significant reduction of IFNγ+CD4+ T cells in the ZAP70 ± group. To note, T cells with partial deletion of ZAP70 had an impaired activation and pronounced apoptotic processes, particularly the intrinsic pathway, which leads to milder joint inflammation (72). NMT1 is an enzyme that attaches the fatty acid myristate to the N-terminal glycine of proteins to sort them into soluble and membrane-bound fractions. Myristoylation-incompetent T cells promote IFNγ and IL-17 production, lead to tissue inflammation and affect T cell differentiation. Experiments performed in-vivo demonstrated that siRNA-mediated NMT1 knockdown induced accumulation of tissue Th1 and Th17 cells, suggesting that an overexpression of this molecule can result in an anti-inflammatory activity with consequent amelioration of the disease (73).

Over the past year, progress has also been made in the area of B lymphocytes. Recently, Mahendra et al. observed higher expression of IL-15Rα, sIL15Rα and AREG (EGFR ligand) on B cells of CCP+ RA patients using RNA-sequencing. Among them, AREG seems to induce FLS proliferation and invasiveness and osteoclasts differentiation from circulating monocytes, in parallel to ACPA activities (74). A peculiar B cell subset, known as CD21-/low DC memory B cells, has been found to correlate to joint destruction in female patients with ACPA+/RF+ RA. This seems to be due to increased expression of RANKL, promoting osteoclasts activity, and of CXCR3, receptor for CXCL9, CXCL10 and CXCL11, leading to the initial infiltration of these cell subsets in the inflamed joints (75).

As far as ACPA are concerned, two studies explored the process behind their reactivity against citrullinated peptides and proteins. They described the cross-reactivity against multiples citrullinated epitopes of the majority of ACPA. Since many of these antibodies had the same germline genes and rearrangements, but displayed different degree of reactivity, these studies linked this different reactivity to somatic hypermutations accumulated over time, with some ACPA displaying a higher light chain somatic hypermutation dependency than the heavy chain (76). Since ACPA+ patients have a worse prognosis with an increased risk of joint destruction, it has been investigated the possibility to induce tolerance in CP-reactive B cells through Siglec-Engaging Tolerance-Inducing Antigenic Liposomes (STALs). A reduced production of ACPA by B cells from RA patients was observed following exposure to CCP-STALs in-vitro, and an impaired ability to produce ACPA in CCP-STALs-treated SJL/J mice in-vivo. These intriguing results suggest the possibility to use STALs-CCP for inducing tolerance and reducing ACPA formation, leading to control RA development and progression (77). However, CP-reactive memory B cells are rare in peripheral blood and therefore their assessment is challenging. To overcome this problem, Germar et al. cloned signalling-competent B cells from RA patients using genetic reprogramming by Bcl6/Bcl-XL transduction for molecular and functional characterisation. Although only three clones were stably maintained, these cells expressed molecular markers related to autoimmunity or specifically to RA, such as CD40, IL7R, C5aR1, co-stimulatory molecules and pro-inflammatory cytokines for a complete presentation, activation and polarisation of T cells. With their approach they provided a useful tool to further define the molecular and functional traits of autoimmune B cells (78). In parallel, Ochi et al. observed the presence in the RA synovia of survival niches that promoted the homing, survival and functional activities of plasmacells. These survival niches appeared to contain both CD14+ my-
eloid cells as well as cells with nurse-like functions responsible for local production of autoantibodies in the joints of RA patients. Thus, targeting specific cell subsets involved in the RA pathogenesis is possible to control the disease development and progression (79).

**Take home messages**

- EV may be useful for monitoring the activation status of total CD4+, total CD8+ and Th1/Th1c1-Type T cells (60).
- The SAMD9 gene expression is a potential T cell activation marker (62).
- A dysregulation of Tfh and an imbalance between Tfh and Tfg may be involved in RA pathogenesis (63, 64, 65).
- The expression of Helios could be a marker for the identification of Treg in RA (68).
- Altered function of CD8+ Tregs may contribute to RA pathogenesis (70).
- AREG expression on B cells may induce FLS proliferation and invasiveness and osteoclasts differentiation in RA (74).
- A peculiar B cell subset (CD21-/low DC memory B cells) may be involved in joint destruction in RA (75).
- STALs-CCP might be used for inducing tolerance and reducing ACPA formation (77).

**Conclusions**

In the last year several studies have been published in order to better understand the pathological mechanisms underlying RA. In particular, genetic and epigenetic studies have allowed us to identify new mechanisms that may at least partly explain the development of the disease. In parallel, interesting and promising results for future development of therapeutic approaches have been reported in the context of the innate and adaptive immune systems. Studies at molecular and cellular levels have clarified some mechanisms that regulate different cells belong to the innate immune system as well particular subsets of lymphocytes involved in the adaptive one, allowing to define more precise targets for novel specific disease-modifying therapies.

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