Review

One year in review 2019: pathogenesis of rheumatoid arthritis

C. Croia[1], R. Bursi[2], D. Sutera[1], F. Petrelli[1], A. Alunno[2], I. Puxeddu[1]

[1]*Immuno-Allergology Unit, Department of Clinical and Experimental Medicine, University of Pisa, Italy;*
[2]*Rheumatology Unit, Department of Medicine, University of Perugia, Italy.*

Cristina Croia, PhD
Roberto Bursi, MD
Donatella Sutera, MD
Fiorella Petrelli, MD
Alessia Alunno, MD, PhD
Ilaria Puxeddu, MD, PhD

Please address correspondence to:
Dr Ilaria Puxeddu,
Department of Clinical and Experimental Medicine,
University of Pisa,
Via Roma 67,
56126 Pisa, Italy.
E-mail: ilaria.puxeddu@unipi.it

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease influenced by both genetic and environmental factors. Over the last few years, particular attention has been given to novel genes and to the close interaction between genetic factors and epigenetic mechanisms. Research has also focused on the influence of environmental factors on disease development, and on new mechanisms of the innate and adaptive immune system that can influence the different stages of RA. However, there are still several aspects of the disease that need further investigation. Shedding some light on the different aspects of RA pathogenesis will help to improve the current diagnostic tools and to identify new targets for the development of disease-modifying therapies. Thus, in this review we summarise the new insights in RA pathogenesis, resulting from literature research data published in the last year.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease influenced by both genetic and environmental factors. Over the last few years, particular attention has been given to novel genes encoding for lymphocyte signalling, affecting molecules or enzymes that are involved in cell regulatory networks and oxidative stress. The development of RA cannot be fully explained by the tight regulation of genetic factors and epigenetic mechanisms. The environment, including smoking, diet, obesity, infections and microbiota have been proposed to trigger the disease in genetically predisposed individuals. The clinical picture of RA is the result of a close interaction between cells, soluble mediators, autoantibodies and signal transduction pathways of the innate and adaptive immune system, all involved at different stages of the disease. In the current review we reported a Medline search of articles in English published in the PubMed database from 1st January 2018 to 31st December 2018. The new insights in the field of RA pathogenesis may improve current knowledge of the disease and contribute to identifying new targets for the development of novel disease-modifying therapies.

Genetic aspects

Genome-wide association studies (GWAS) and independent replication studies identified potential genes associated with RA susceptibility, especially major histocompatibility complex (MHC) genes, divided into class I (HLA-A, B, C), class II (HLA-DR, DP, DQ), and class III sub-regions. It has been established that HLA-DR, especially HLA-DRB1 locus, provides important genetic contribution for the risk of development of RA, by encoding the MHC class II antigen-presenting molecules which can accommodate a widely range of peptide ligands. So far, it is well known that most of the RA-associated HLA-DRB1 alleles share similar amino acid sequences at position 70–74 on the HLA-DR β chain, named shared epitope (SE), which is highly prevalent among anti-citrullinated protein antibodies (ACPA)-positive patients. By investigating the interaction between RA associated antigens and HLA-SE allomorphs, Ting *et al*. identified a preferential binding of three HLA-DRB1 allomorphs (HLA-DRB1*04:01/*04:04/*04:05) by self-antigens implicated in RA. In particular, a strong correlation between binding affinity and citrullination at P4 of the bound peptide ligand was reported for all three HLA-DRB1 allomorphs. However, we have to take in account that not all HLA-DRB1 allomorphs were shown to bind citrullinated antigens with the same affinity, probably

due to β-chain polymorphisms residing outside the SE motif (1). Furthermore, the SE alleles are associated with RA susceptibility as well as with the severity of the disease, and they can help to identify different genetic profiles in subsets of RA patients. HLA-DRB1 analysis performed in different RA subgroups, allowed the identification of a genetically unique subset of RA patients and distinct genetic background (2). In addition to the well-recognised role of HLA-DRB1, increasing attention has recently been given to HLA-DP genes for their role in infection processes and autoimmunity. In this regard, single-nucleotide polymorphism (SNP) rs9277535 in HLA-DPB1, a subunit of HLA-DP, was shown to be strongly associated with RA susceptibility in a West Chinese population (3).

Beyond HLA loci, a number of non-HLA genes have also been implicated in the susceptibility to RA. For example, polymorphisms in the promoter regions of cytokine genes may modulate cytokine expression, thus affecting disease susceptibility. It is recognised that IL-1β plays a key role in mediating joint inflammation and destruction in RA, and the role of polymorphisms of the IL-1β gene within the promoter region -511(C/T) has been recently investigated in RA. The mutant allele (T) of IL-1β-511 promoter SNP was reported to be associated not only with elevated anti-CCP and IL-1β levels, but also with disease susceptibility in a cohort of North Indian RA patients (4). Among members of IL1 gene family, the IL37 gene, particularly IL37 gene rs3811047 SNP, was found to be associated with more severe disease activity in an Egyptian RA population (5). In parallel with the IL1 family genes, polymorphisms of IL10 gene have been also investigated, since IL10 exerts immunoregulatory as well as anti-inflammatory functions in the rheumatoid synovial. Also, an association of IL10-1082 A/G promoter SNP with the susceptibility to RA has been reported in a North Indian population (6). Genetic variants in TNF superfamily genes are also under investigation due to the key role of their encoded proteins in disease pathogenesis. For instance, TNF-like ligand 1A (TL1A), encoded by TNF superfamily member 15, located on chromosome 9q32, is known to exert pleiotropic effects on cell proliferation and activation as well as on differentiation of immune cells, such as T helper cells. In this regard, the TL1A rs3810936 and rs7848647 polymorphisms were shown to be protective genotypes against the development of the disease (7). In addition, both TNF receptor and TNF receptor-associated factors have been also extensively investigated in RA. TNF receptor-associated factors (TRAF) were initially identified as adaptor proteins for TNF receptor (TNFR) family in the signalling of intracellular pathways. However, they were subsequently shown to be signal transducers for a wide variety of receptors that are involved in regulating cell death and survival and cellular responses to stress. Within the TRAF family, TNF receptor associated factor 6 (TRAF6) was found to induce NF-κB activation, resulting in the transcription and secretion of a variety of inflammatory mediators, involved not only in synovial inflammation, but also in cartilage and bone destruction. In addition, TRAF6 rs540386 SNP has been recently associated with low bone mineral density (BMD) in a cohort of RA patients (8).

Besides cytokines and growth factors, chemokines play an important role in the inflammatory state of RA and SNP of chemokine-encoding genes have been extensively studied in RA. For instance, the chemokine C-C motif ligand 4 (CCL4), secreted under mitogenic signals and acting as a chemo-attractant factor for different immune cells, was widely investigated. Analysis of SNP of the *CCL4* gene showed that the nucleotide T over the rs1719153 is associated with decreased *CCL4* gene expression with consequent decreased risk for RA development (9).

Over the last few years, particular attention has been given to novel genes encoding for lymphocyte signalling regulating molecules or enzymes that are involved in cell regulatory networks and oxidative stress. In this context, CD226, a 67-kDa type I transmembrane glycol-protein encoding by CD226 gene, is expressed on immune cells such as natural killer (NK) cells, T lymphocytes and monocytes. Its role is primarily linked to intercellular adhesion and lymphocyte signalling and the T allele of the CD226 gene has recently been identified as a potential susceptibility risk factor for the development of RA in a cohort of Egyptian patients (10).

In parallel, particular attention has been given to Caspases (CASPs), a family of cystein proteases that are known to regulate apoptotic signalling (apoptotic CASPs) or to mediate host defence against microbial infection and processing pro-inflammatory cytokines (inflammatory CASPs). The latter, have been widely investigated in inflammatory diseases such as intestinal bowel diseases and in neoplastic conditions. Only recently has their involvement in autoimmune diseases been proposed. Interestingly, among the main genes encoding the inflammatory CASPs (CASP1, CASP4 and CASP5), the CASP5 rs9651713 polymorphism has been correlated with an increased risk of RA in a Chinese population, supporting the involvement of these enzymes in the pathogenesis of RA (11).

Increased oxidative stress, decreased antioxidant levels, and impaired antioxidant defenses might occur during RA pathogenesis. In particular, Paraoxonase (PON) is a group of proteins present in three forms (PON1, PON2, PON3) encoded by genes *PON1, PON2*, and *PON3*, located on the long arm of chromosome 7 between q21.3 and q22.1. Among them, *PON1* and *PON3* are plasma enzymes, structurally and functionally related to HDL, while *PON2* is characterised by an intracellular location. A positive association between PON1 L55M (rs854560) polymorphism and an increased severity of the disease was observed in RA patients with moderate or high activity of the disease in the presence of anti-CCP antibodies. Furthermore, the polymorphisms of PON1 L55M (rs854560) and Q192R (rs662) act in synergy in increasing disease activity in anti-CCP-positive patients and in those patients with moderate or high active disease (12).

Although HLA and non-HLA loci are

well recognised to be involved in RA, they cannot explain disease susceptibility alone. A fundamental contribution in understanding the mechanisms of gene modulation comes from epigenetic studies. This field deals in particular with inheritable and potentially reversible modification of DNA which can modulate gene expression without altering DNA sequence and which may be influenced by environmental factors. Among the distinct epigenetic mechanisms, growing interest has recently been given to the possible contribution of DNA methylation in RA pathogenesis. Through genome-wide analysis of DNA methylation in disease-discordant monozygotic twins, Webster *et al*. identified a differentially variable DNA methylation signature in the absence of differential methylation, supporting the importance of epigenetic variability in the pathogenesis of RA as well as of other autoimmune diseases (13). Novel methodologies have been developed in order to better characterise the epigenomic landscape in RA, in particular in fibroblast-like synoviocytes (FLS), structural synovial cells that play active role in the inflammatory and remodelling processes. In order to cluster the RA genome into regions with similar epigenomic profiles, a large collection of epigenomes for RA FLS has been generated by profiling six histone modification patterns, open chromatin regions, RNA expression and whole-genome DNA methylation. Subsequently, these epigenomic data have been integrated into a single analysis. It was therefore possible to identify several RA-specific pathways, some of which are unexpected. In particular, the "Huntington's Disease Signalling", specific to the Huntingtin-interacting protein-1, seems to modulate the FLS capacity to invade the extracellular matrix (ECM), contributing to tissue remodelling. These intriguing results allowed the introduction of novel diagnostic approaches and the identification of new potential targets for the development of further treatments (14). In addition to DNA methylation, the evaluation of histone modifications may be useful to understand the epigenomic profile in RA subtypes. These may represent good epigenetic indicators of chromatin state associated with gene activation or repression. In this regard, aberrant histone lysine methylation (HKM) has been reported in RA FLS. Specifically, the dysregulated gene expression of HKM-modifying enzymes, including histone lysine methyltransferases and demethylases, have been implicated in the alteration of HKM, leading to changes in the gene expression of FLS, contributing to the amplification and perpetuation of inflammation and remodelling of RA synovia (15).

Another class of heredity variants that has recently been the objective of attention is the class of DNA polymorphisms affecting microRNA (miRNA). These belong to a class of small, non-coding RNA molecules with approximately 21 nucleotides in length that can regulate gene expression by reducing the ability of their target messenger RNA (mRNA) to promote proteins synthesis. Altered miRNA expression has been reported in RA patients and SNP in miRNA have been investigated in humans and in animal models. For instance, SNPs of miRNA-146a (rs2910164 and rs2710164) and miRNA-499 (rs3746444) have been associated with RA susceptibility in an Egyptian population (16) (17). In parallel, Fernandes *et al*. have analysed miRNA and gene expression profiles in peritoneal cells of the two mouse lines AIRmax and AIRmin, which differ in their susceptibility to experimental arthritis after pristane injection (Pristane-induced arthritis model) (PIA). This is a mouse model resembling histopathological, immunological and clinical aspects of RA. They demonstrated that cells from susceptible AIRmax mice had higher gene and miRNA modulation than cells from resistant AIRmin mice, suggesting that miRNA expression may be responsible for the different PIA susceptibility in these strains. Moreover, miR-132-3p/212-3p, miR-106-5p, miR-27b-3p and miR-25-3p were the miRNAs with the highest expression in susceptible animals (18). Moreover, the down-regulation of MiR-338-5p, involved in the initiation, progression and metastasis of many human cancers, seems to be implicated in the pathogenesis of RA and in the development of the disease (19).

Environmental factors

Cigarette smoking

The environment, including smoking, diet, obesity, infections and microbiota have been proposed to induce the development of RA in genetically predisposed individuals. Cigarette smoking (CS) is the most known external factor identified as trigger of RA (20). The direct effect of cigarette smoke condensate (CSC) on the development of arthritis has recently been demonstrated in the CIA model. Inoculating intraperitonally of CSC one day before immunisation of the animal model was sufficient to promote arthritis. Therefore, the analysis of a single CSC compound using sequential fractionation, silica gel column chromatography, Mass Spectrometer and the Gas Chromatograph, revealed that the compound potentially relevant for inducing arthritis was 5-hydroxy-2-methylpyridine (5H2MP) which was not mutagenic and did not exert its activity via aryl hydrocarbon receptor (AHR). The authors hypothesised that this effect may be the result of an amplification of the AHR pathway or even of an independent mechanism with production of an active metabolite. Activation of this receptor seems to play a key role in the pathogenesis of RA and in CS-induced arthritis. Chen *et al*. observed that the AHR and its downstream gene expression were present in both smoking and non-smoking rheumatoid peripheral blood mononuclear cells (PBMC) with significantly higher expression in smoking patients (21). Therefore, administrating AHR pathway agonists to transgenic mice carrying human SE-coding alleles resulted in a robust increase in arthritis severity, bone destruction, overabundance of osteoclasts, and IL17-expressing cell infiltration in the inflamed joints, providing new insights into the well-described, but poorly understood amplification of the genetic risk for RA upon exposure to environmental pollutants (22).

The molecular mechanisms underlying smoking-induced arthritis were further investigated. By using *in-vivo* model of IL17ra KO mice and *in-vitro* systems,

Talbot *et al*. found that smoking is able to exacerbate arthritis and to increase Th17 differentiation of T cells via AHR activation (23).

Among the CSC that might contribute to the induction of arthritis, Hydroquinone (HQ) was recently identified as an active player in the early phase of the disease, by increasing the infiltration of inflammatory cells, the levels of IL6 in the synovial fluid, the deposition of ECM proteins, proliferation of synoviocytes and by promoting higher frequency of AHR and neutrophils expressing IL17 in the synovial membrane (24).

CS seems to be involved in RA not only for its role in inflammatory and remodelling processes, but also for its capacity to influence autoantibodies production. It has been observed that a subset of patients with anti-Peptidyl arginine deiminase 4 (PAD4) antibodies that cross-react with PAD3 (anti-PAD3/4) are at the highest risk for interstitial lung disease, and this risk is increased by a history of CS. Therefore, Cappelli *et al*. aimed to investigate whether smoking is aetiologically linked to the development of anti-PAD4 antibodies, but they did not demonstrate in the RA patients any association of anti-PAD4 antibodies with smoking, suggesting that other environmental factors might be involved in these processes (25).

Among environmental factors, hypoxia seems to be a prominent feature in RA, by modulating the activity of FLS via hypoxia-inducible factor 1α (HIF1α). Interestingly, Yu *et al*. have discovered that hypoxia is able to promote RA development not only because it induces inflammation and angiogenesis, but also because it induces the process of citrullination in human FLS. In particular, they found that PAD2 and citrullinated proteins were increased in human FLS after exposure to hypoxia and this was down-regulated by knocking down HIF1α using HIF1α siRNA (26).

Obesity and diet

An association between RA and obesity has been demonstrated, but the precise mechanisms involved have not yet been elucidated (27). A meta-analysis based on over 350,000 patients showed that higher body mass index (BMI) increases the risk of RA compared to normal weight, mainly in the female population (28). Adipokines are key players in the activity of the adipose tissue and they seem to exert direct effects on the immune system. Among adipokines, it has recently been proposed that leptin plays an active role in RA with higher levels detected in the circulation of RA patients compared to healthy controls. Wang *et al*. demonstrated that in RA patients leptin was able to increase the number of circulating follicular T helper cells, the levels of IL6, IL21 and IL12, mainly through activation of STAT1 and STAT3 pathways (29). While leptin seems to be involved in the inflammatory process, adiponectin seems to increase bone erosions in the CIA model. This may be due to the induction of osteopontin expression by synovial FLS, which recruits osteoclasts with consequent bone erosion (30). The role of adiponectin in RA was also studied in humans, particularly in first-degree relatives of subjects at high risk of RA who have not yet developed the disease (31). The chemokine C-X-C motif chemokine ligand 5 (CXCL5), also known as epithelial neutrophil activating peptide 78 (ENA78) represent a link between obesity, inflammation and insulin resistance (IR) in the general population. Since CXCL5 has also been found to play a role in the inflammatory process of RA, and chronic inflammation promotes IR with impairment of pancreatic β-cells in RA patients, Tejera-Segura *et al*. have hypothesised a role of this chemokine in the development of IR in RA. However, they showed that in RA patients, unlike the general population, the CXCL5 is not related to IR, suggesting that other mechanisms are involved in the development of IR in RA (32). It has also been hypothesised that the possibility of non-response to RA treatment might be linked to metabolic disorders and that the treatment of these disorders might exert positive effects on RA as well. In fact, Metformin, a biguanide anti-diabetic drug mainly used in type 2 diabetes, exerts therapeutic efficacy in other diseases besides type 2 diabetes, such as experimental autoimmune arthritis and colitis by reducing STAT3 activation. Kim *et al*. have recently shown in the CIA model that Metformin was able to ameliorate the development of arthritis in obese mice by reducing autoantibodies expression and joint inflammation. Thus, these results strongly support the beneficial effect of this treatment in RA when it is associated with metabolic disorders (33).

Recently, diet and nutritional supplements has been the subject of investigation for their protective roles in several pathological conditions. The Mediterranean diet is commonly known as one of the healthiest dietary patterns existing, influencing the gut microbioma and indirectly modulating the immune system. This diet regime seems to exert beneficial effects on RA, mainly through the intake of antioxidant elements such as vitamin C, retinol and omega-3 polyunsatured fatty acids (PUFA). In addition, high dietary quality with a high intake of fish, whole grains, fruit and vegetables and low intake of meat and sweets seems to be negatively associated with inflammatory markers such as CRP and ESR (34). Erythrocyte levels of the n-6 PUFA linoleic acid are inversely associated with risk of RA, whereas no associations were observed for other n-6 or n-3 PUFA (35). Up to now, it is not known which component of the Mediterranean diet is responsible for their beneficial effect on RA. Recently, particular attention was given to the monounsaturated fatty acid (MUFA), an important component of this diet. Daily MUFA intake might suppress disease activity in RA patients, supporting the potential benefits of the components of the Mediterranean diet in controlling RA (36).

Microbiota

The inflammatory state underlying RA seems to be closely linked to the microbiota. Many studies suggest that a chronic inflammatory response induced by gut dysbiosis can critically contribute to the pathogenesis of some autoimmune diseases. Diet can modify intestinal microbiota and can influence intestinal barrier strength, integrity and permeability. We know that the microbiota of RA patients differs from the

general population and that anti-rheumatic drugs can exert positive effects on its regulation. Recently, analysis of the components of microbiota enabled a better understanding of the link between inflammation and microbiota. In particular, Muniz Pedrogo *et al*. demonstrated an increase of *Clostridiacee* in the stool samples of RA and inflammatory bowel disease-associated arthropathy, suggesting a potential common microbial link between the two forms of arthritis (37). Interestingly, both gut microbiota and arthritis have been studied in the murine model of CIA in which broad-spectrum antibiotics were administered 7 days before or 21 days after immunisation with type II collagen (38). Interestingly, a depletion of the microbiota prior induction of CIA resulted in reduction in disease severity by 40% with a decrease in circulating inflammatory cytokines. In parallel, a delay in the production of IL17A and IL22 in intestinal tissue has been observed. In the other group of mice treated 21 days after immunisation, a greater reduction in severity was even observed, suggesting that intestinal dysbiosis is able to trigger mucosal immune responses. In addition to the commensal bacteria and their pathogenic components present in the gastrointestinal tract, the bacteria present in the oral cavity exert pathological activity in autoimmune diseases. An interesting study showed that multiple environmental pathogens contribute independently or concomitantly to reaching worse disease activity, and to affecting the outcomes of RA (39). Subsequently, Ceccarelli *et al*. analysed tongue *Porphiromonas (P) gingivalis* presence and quantification in a large cohort of healthy subjects and RA patients, comparing these data to those obtained in patients with periodontitis, knee osteoarthritis or fibromyalgia. They showed a similar prevalence of *P.gingivalis* in RA and periodontitis, with higher prevalence in these groups compared to the control group. In addition, the *P.gingivalis*/total bacteria rate was lower in RA patients in the remission phase of the disease and was positively correlated to the disease activity (40).

Infections

In parallel to the gut microbiota, several viral and bacterial infections seem to be responsible for the development of autoimmune diseases, including RA. Arleevskaya *et al*. characterised the infection events in a longitudinal cohort of first-degree relatives of patients with RA, evaluating their associations with the development of the disease (41). Interestingly, they observed that an annual increase of respiratory tract infections was found at the pre-clinical stage of RA, probably due to alteration in the immune system, resulting in susceptibility to infection. The authors also hypothesised that the infectious events can predispose to the development of RA. In particular, the role of Epstein-Barr virus (EBV) in the pathogenesis of RA has been demonstrated, although some aspects need to be better defined. Recently, Kuusela *et al*. have investigated the prevalence of asymptomatic activation of EBV in RA patients and the correlation of serum EBV DNA with disease activity. Interestingly, they observed that active RA was associated with a lytic EBV infection which may play an active role in the pathogenesis of RA (42). Subsequently, to better characterise the influence of EBV infection in the development of RA, Masuoka *et al*. evaluated the EBV gene in the synovial tissue of RA patients by using nested PCR for the amplification of EBV nuclear antigen-1 (EBNA-1). Interestingly, they observed that EBV infection can contribute to the onset of RA and chronic inflammation in synovial tissue and that the frequency of EBNA-1 gene variants was low and not significantly different between RA and control groups, suggesting that EBNA-1 gene variants are not a risk factor for RA. In addition, they demonstrated that HLA-DRB1 with SE was a genetic risk factor for the development of RA, but neither the presence of EBV nor EBNA-1 gene variants differed between SE-positive and SE-negative patients. According to their results, it is possible to hypothesise that these two risk factors, SE and EBV, may be independent and not related to each other (43).

Molecular mimicry among EBV, *Mycobacterium avium paratuberculosis* (MAP) and host peptides has been considered as an RA pathogenetic mechanism. By using bio-informatic analysis, Bo *et al*. identified high sequence homology among interferon regulatory factor 5 (IRF5), EBV antigen BOLF1 and MAP antigen MAP_4027. They have also evaluated in the sera of RA patients the presence of antibodies directed against human homologous IRF5 cross-reacting with BOLF1 and MAP_4027. According to their results, they suggested that IRF5 might be a potential target of RA, supporting the hypothesis that EBV and MAP infections may be involved in the pathogenesis of the disease, leading to a secondary immune response, cross-reacting against RA self-peptides (44).

Innate immune response

The innate immune system plays a key role in non-specific recognition of pathogens and represents the first defence against viruses or bacteria. Following the identification of microbial substances, the phagocytosis process is activated by innate immune cells with the release of specific cytokines and chemokines at the site of infection. Several cells of the innate immune system are involved, including macrophages, monocytes, dendritic cells, neutrophils, natural killer cells, mast cells, eosinophils, innate lymphoid cells (ILCs) and myeloid-derived suppressor cells (MDSCs). All these cells act through a large number of specialised receptors, such as pattern recognition receptors (PRRs) expressed on different immune cells. Among PRRs, the Toll-like receptors (TLRs) are able to recognise several microbial epitopes on pathogens with subsequent activation of the inflammasome.

Several studies have thoroughly investigated the role of monocytes in RA pathogenesis, recognising the role of these cells in the joint inflammation and bone erosion. Smiljanovic *et al*. have recently analysed the role of monocytes in RA by using transcriptome technology and by performing a cytometric profiling of monocytes from bone marrow (BM) and from peripheral blood (PB) of RA patients. The main pattern found in both RA-BM and RA-PB was similar

to the one of less mature and differentiated precursors, *i.e.* the "left-shift pattern". Based on these observations the authors suggested an increased turnover of RA monocytes, which migrate from BM into the blood stream and from the circulation into the inflamed synovia. The authors confirmed the transcriptome data in RA by cytometry analysis and showed that monocytes are activated in the joint tissue, thereby supporting the relevant role for local disease-specific stimuli (45). It is well known that monocytes can differentiate into M1 macrophages with a pro-inflammatory phenotype or into M2 macrophages with an anti-inflammatory phenotype. Fukui *et al.* showed that in RA patients M1/M2 ratio positively correlate with the rate of differentiation of osteoclasts. In particular, they found that in ACPA-positive patients the M1/M2 ratio is higher than in ACPA-negative patients and that a higher M1/M2 ratio also had a higher levels of ESR and CRP, suggesting that the modulation of M1 and M2 subsets could represent a potential therapeutic target in RA (46). Several soluble mediators orchestrate monocyte recruitment in the inflamed tissue. In particular, Cecchinato *et al.* proved that high mobility group protein (HMG) B1, a damage-associated molecular pattern (DAMP) released by necrotic cells after tissue injury, and the chemokine CXCL12, might act in synergy, increasing the migration of monocytes to the site of inflammation and promoting the progression and amplification of the inflammatory processes. Monocytes purified from RA patients in clinical remission compared to those from RA patients with an active disease, require 10-fold less concentration of HMGB1 to enhance CXCL12-induced monocyte migration. Therefore, it has been hypothesised that the activity of this heterocomplex might depend on COX2 and JAK/STAT pathways and are affected by the redox potential of the microenvironment. Thus, modulation of the heterocomplex CXCL12/HMGB1 might be an alternative tool in RA patients that poorly respond to conventional treatments (47). Since monocytes are important players in local inflammation and bone erosion, down-regulation of monocytes activation can be another possible way of inhibiting inflammation and bone erosion in RA. It has been shown that FLS from RA synovia expressed higher levels of CXCL12, and the over-expression of this chemokine would in turn increase the recruitment of monocytes via CXCR4. Thus, once monocytes migrate to the synovia, they differentiate into pro-inflammatory macrophages, releasing several cytokines and chemokines that contribute to the amplification and perpetuation of local inflammation and tissue damage (48). Thus, the recruitment of monocytes into the joint of RA patients can be regulated by the interaction between monocytes and FLS in the inflamed tissue. In particular, it has recently been demonstrated that the disintegrin and metalloprotease ADAM17 might play an important role in monocyte-FLS interaction. The levels of this mediator are increased in both the serum and synovial fluid of RA patients. In addition, ADAM17 is expressed in FLS and knocking down this molecule leads to a drastic reduction in the adhesion of monocytes to FLS (49). Besides ADAM17, circulating immune complexes (ICs) purified from RA patients, but not those from healthy donors, were able to promote a pro-inflammatory phenotype in monocytes with an over-production of TNF-α in response to a variety of innate stimuli (50).

Recently, Masako *et al.* evaluated the impact of different monocyte populations on disease activity in RA. By flow cytometry analysis they divide the monocytes into CD14brightCD16-, CD14brightCD16$^+$ and CD14dimCD16$^+$ subsets and they observed that the CD14brightCD16$^+$ subset is increased in the PB of RA patients, but decreased following anti-IL6 or anti-TNF treatment together with the decrease of disease activity (51).

As previously reported, circulating monocytes can infiltrate inflamed joint in RA and differentiate into M1 and into M2 macrophages. Interestingly, Yoon *et al.* investigated the metabolic reprogramming of monocytes/machrophages expression, since their participation in a specific immune response is an energy-demanding process and consequently it requires a strict regulation of their metabolic programmes. They observed that RA monocytes express a higher level of SLC7A5, a key regulator of the uptakes of different amino acids, and that the SLC7A5-mediated leucine uptake promotes the production of pro-inflammatory cytokines in human monocytes/macrophages. By blocking SLC7A5, IL1β is reduced and this effect seems to be due to the inhibition of the leucine-mediated activation of mTORC1, the master regulator of cellular metabolism (52).

Several *in-vivo* models have been used to analyse the role of macrophages in RA. Hagert *et al.* developed a new mouse model of chronic arthritis by injecting 4 monoclonal anti-type II collagen antibodies followed by mannan injection in RA B10.Q mice. Interestingly, they found that this model developed the disease independently of the contribution of T and B lymphocytes. Instead, macrophages and the complement cascade seem to play crucial roles in driving RA in this model. The authors suggest that mannan is able to activate macrophages via TLR4, suggesting that the innate immunity can induce a chronic active arthritis, also without the involvement of the adaptive response (53).

An interesting discovery linking macrophages, fat tissue and the mechanisms involved in RA pathogenesis has been reported by Giles *et al.* Adipose tissue macrophages (ATM) are a strong source of pro-inflammatory cytokines, regulating the functions of the adipose tissue. The authors demonstrated for the first time that adipose tissue from RA patients show 76% more macrophages than adipose tissue from normal patients. In addition, RA patients have 1.5-fold higher number of crown-like structure, formed by ATM surrounding dead adipocytes and secreting inflammatory cytokines, with association with IR. Importantly, among RA patients there was a striking correlation between the levels of ATM and/or crown-like structures, systemic inflammation and autoantibodies production. Moreover, levels of ATM and/or crown-like structures were significantly lower in pa-

tients using methotrexate, leflunomide, TNF inhibitors or statins, suggesting that adipose tissue may be the target of pharmacological therapies used in RA (54). Besides monocytes and macrophages, neutrophils play important roles in the immune system and they represent around 80–90% of the total cells in RA synovial fluid/joints. These cells have been linked to the pathogenesis of RA for their release of pro-inflammatory cytokines, reactive oxygen species (ROS), granules containing degradative enzymes and neutrophils extracellular traps (NETs) which can cause further damage to the tissue and amplify the neutrophil response. Like any other cells, neutrophils can undergo autophagy, a self-protective mechanism where the cell disassembles and gets rid of unnecessary or dysfunctional components. Recent studies have reported that autophagy is involved in the pathogenesis of RA, but the clear mechanisms have yet to be elucidated. Qiyuan *et al*. found that neutrophils undergo a higher rate of autophagy in RA synovia as indicated by the increase in the autophagy-related protein LC3 and a lower lysosomal PH in neutrophils. Several cytokines such as IL6, IL8, IL10 and MCP1 were higher in RA synovial fluid and correlate with the autophagy rate, suggesting that these cytokines are possible driving factors for neutrophil autophagy. In addition, this process may be regulated by different signalling pathways, including those of IL17 (55).

A fascinating study by Rhys *et al*. has investigated the potential role of neutrophils microvesicle on the inflammatory activation of macrophages. By combining *in-vitro* and *ex-vivo* techniques, the authors demonstrated that a specific subset of neutrophils microvesicle (expressing annexin A1 and phosphatidylserine) can attenuate macrophages activation in response to LPS and IFNγ. In co-culture experiments, the authors also demonstrated that classically activated macrophages stimulated with neutrophil-derived microvesicles no longer activate adjacent fibroblasts, suggesting that modulation of neutrophil vesicles might be a new therapeutic approach for joint disease (56). Recently, neutrophils have been linked to the formation of ACPA in RA. Gorlino *et al*. found that in the presence of ACPA in the synovial fluid of RA patients, the number of neutrophils was increased in the joint and correlates with the levels of IL8, the main chemokine involved in neutrophil migration. By *in-vitro* and *in-vivo* studies, they also demonstrated that higher synovial ACPA levels induce ROS production, DNA extracellular release and the induction of ICAM-1 expression in neutrophils (57). Subsequently, by studying the binding of ACPA-IgG IC to individual Fc gamma receptors (FcγR), Kempers *et al*. identified a pivotal role for the neutrophil-expressed FcγRI receptor in binding the ACPA-IgG IC. Since neutrophils isolated from the synovial fluid exhibit an activated state and express FcγRI, this strongly suggests that the binding of FcγRI to IC could contribute to local inflammatory processes and therefore to the mechanisms underlying the pathogenesis of RA (58).

Adaptive immune response

Different studies have demonstrated that a dysregulation of adaptive immune response plays an important role in the pathogenesis of RA and that several mediators and cellular components may be involved. Among the adaptive immune cells, T and B lymphocytes might contribute to the pathogenesis of RA at different levels. Liu *et al*. have discovered that β2-adrenergic receptor (β2-AR) expressed by $CD4^+$ T cells is reduced in the ankle and in the spleen of CIA model, compared to the healthy mice. In mice with CIA, Th17 cells, the Th17-related transcriptional factor retinoic acid related orphan receptor-gt (ROR-gt) and also IL17 and IL22 cytokines, are more consistently present compared to healthy mice. Treatment of these mice with norepinephine (NE), which signals through the β2-AR expressed by lymphocytes, blocks Th17 cell differentiation and consequently reduces ROR-gt, IL17 and IL22 production, ultimately leading to an anti-inflammatory microenviroment. Treating $CD4^+$ T cells from CIA mice with β2-AR agonist terbutaline (Terb) is able to induce the same effects, along with the inhibition of $CD4^+$ T cell proliferation. β2-AR mediates all these effects through a cAMP-protein kinase A signalling, and β2-AR antagonist ICI118551 prevents the effects of NE and the PKA inhibitor H-89 blocks the effects of Terb, suggesting new potential therapeutic approaches via modulation of β2-AR. (59).

IL-34 does not directly influence Th17 $CD4^+$ T cells from RA patients, however, if they are co-cultured with stimulated monocyte-like cell line (THP-1) in the presence of anti-CD3/CD28 antibodies, it can stimulate THP1 CSF1R. This treatment induces the up-regulation of ROS production which, in turn, leads to increasing the release of IL6 with the consequent enhancement of the Th17 cell generation. In this co-culture system, the levels of the pro-inflammatory mediators TNF-α and IL1β are up-regulated, indicating their possible role in Th17 generation. The use of TNFRII antagonist, but not of IL1βR, can reduce ROS production from THP1 and $CD4^+$ T cells and consequently the release of IL6 and the formation of Th17 (60). Recently, It has been demonstrated that IL-17-producing $CD4^+CD161^+$ T cell subset can also play a role in RA pathogenesis. The level of $CD4^+CD161^+$ T cells, along with their level of expression of CD98 and CD147, is significantly increased in the synovial fluid of RA patients compared to the PB. $CD4^+CD161^+$ T cells in the synovial fluid can be used as an indicator of RA disease activity since their expression of CD98 and CD147 is directly correlated with the disease activity index (DAS) on 28 joints. CD147 expression is also associated to the levels of the CRP, thereby suggesting a potential function of this factor in T cell behaviour in local inflammation (61).

The presence of the PTP22 risk allele is associated with several autoimmune diseases, including RA. Naive human $CD4^+$T cells homozygous for that allele over-express a set of genes which are important for cytotoxic T cell differentiation. Moreover, the protein expression of the T-box transcription factor eomesodermin (EOMES) is increased

in T cells from healthy donors homozygous for the PTPN22 risk allele and correlates with a decreased number of naıve $CD4^+$ T cells. In PTPN22 risk allele carriers an accumulation of $EOMES^+CD4^+$ T cells in synovial fluid of RA patients was observed, together with a more pronounced production of perforine (62).

It has also been observed that in RA patients the proportion of Th17 cells and the chemokine CCL20, ligand of CCR6, is higher in the mononuclear cells from the synovial fluid than in those from the PB, while Th1 cells manifest the opposite behaviour. Th17 cells display higher levels of RORγt and CCR6 expression compared to the cells from healthy subjects and osteoarthritis patients, and this is particularly evident in the synovial fluid. Thus, It has been hypothesised that this can lead to a selective increase of Th17 cells migrating to the inflamed joint. In addition, treatment with anti-rheumatic drugs such as methotrexate for 24 weeks is able to reduce the presence of RORγt and CCR6 in Th17 cells, as well as CCL20 expression in the synovial fluid of RA patients, thereby reducing the migration of Th17 cells to target tissues. Surprisingly, this treatment is also able to increase the levels of the chemokine CXCL10 in Th1 cells.

Taken together, these results support the hypothesis that the CXCR3-CXCL10 axis and RORγt -CCR6-CCL20 axis may play important roles in the pathogenesis of RA and the latter could represent a potential therapeutic target in RA (63).

In addition, the *in-vitro* stimulation with IL2 of either PBMC or purified $CD4^+$ T cells, but not of lymph-node derived T cells from healthy subjects and RA patients promotes a significant increase of $Foxp3^+CD25^+CD4^+CD127$ low regulatory T cells (Treg) cells which is not observed if cells are only pre-treated with IL2. Conversely, the rise in Treg cells after treatment with low dose of IL2 could be related to an unmasking of latent Treg cells already present in the PB (64). Recently, it has been demonstrated that in RA the control of auto-reactive cells is defective and that Treg cells have an impaired function. Zafari *et al*. analysed the epigenetic modulation of the Foxp3 Treg-specific demethylated region (TSDR) and Helios gene expression to determine Treg cell alteration in RA patients. Interestingly, they demonstrated that both epigenetic modifications and Helios gene expression may have important roles in the pathogenesis of RA through their effects on Foxp3 gene expression (65).

The dysregulation of the Treg cells in the RA was also supported by recent studies by Wang *et al*. They showed that an increase of miRNA, in particular the exosome miRNA, a negative regulator of cellular gene expression, can downregulate the differentiation of Treg cells in RA patients compared to healthy subjects. In particular, they discovered that the miRNA 17 can suppress the induction of Treg by inhibiting the expression of the receptor II of TGFβ (TGFBRII) (66). In support of these studies, it was also reported that $CD3^+CD4^+CD25^+CD127$low Treg cells increased in the PB of RA, together with an increased level of IL6, TNF-α, IL37 and the cell immunoglobulin and mucin-domain containing-3 (TIM-3). However, no association with disease activity was found, suggesting that this dysregulation may be involved in the mechanisms that regulate the pathogenesis of RA, but are not implicated in the disease activity (67). By analysis of T cell subsets in RA, it has been observed that $CD4^+$ $Tim\text{-}3^+/PD\text{-}1^+/CD4^+$ T cell subset is higher in the circulation of RA patients compared to healthy subjects (68). In addition, a role of PD-1, one of the main immunosuppressive co-stimulatory molecules, which mediates an inhibitory effect, has been also demonstrated. In fact, Luo *et al*. found that the expression of PD-1 on T cells in the PB of RA patients was increased significantly in subjects with a high RF titre, high levels of inflammatory markers and a high DAS28. The expression of PD-1 on T cells in the synovial fluid of was also increased significantly in those RA patients with a high DAS28, indicating that the expression of PD-1 on T cells might be closely linked to the activity of the disease (69).

Subsequently, to support the role of PD-1, Matsuda *et al*. showed that over 60% of synovial $CD3^+$ cells in RA express CD3 which is otherwise undetectable in osteoarthritis and that the majority of the RA synovial lining cells express PD-L1, one of PD-1 ligands. The expression of this ligand is significantly correlated with the amount of synovial T cells, inflammatory markers such as CRP, autoantibodies such as RF and Krenn's synovitis score, supporting the idea that the expression of PD-L1 may reflect the state of the disease (70).

The infections are connected to RA not only for the close interaction with the innate immune system, but also for their possible interactions with the adaptive one. Autoantibodies are produced in lymphoid tissue where lymph node stromal cells (LNSCs) regulate lymphocyte function. Infections can alter the interaction between LNSCs and lymphocytes resulting in defective immune responses. Interestingly, Hanhlein *et al*. showed that upon TLR3 triggering, human LNSCs from RA-risk individuals and RA patients produce fewer T cell guiding chemokines as well as the antiviral molecule MxA, compared with healthy controls, suggesting that during systemic autoimmunity human LNSCs attempt to inhibit immune responses when activated through TLR3 (71).

Analysis of transcription factors related to CXCL13-producing $CD4^+$T cells at local inflammatory sites has highlighted the role of SOX 4 in RA. In fact, it has been shown that this transcription factor is more consistently expressed in $CD4^+$T cells from the synovial fluid of RA compared to the PB and that it can induce the production of the chemokine CXCL13 in the $PD\text{-}1^{hi}CXCR5^{-}CD4^+$ T cell subset *in-vitro*. This effect is particularly evident in a pro-inflammatory environment, contributing to the development of ectopic lymphoid-like structures in the inflamed tissues. At the same time, SOX 4 can have a role in the commitment of Th1, but not of Th2 cells, by increasing the production of IFNγ and the expression of CXCL13 under Th1-polarising condition. To note, the effect of SOX 4 has not been observed in mouse models of RA, opening the way to new stud-

ies to better understand the difference in the regulation of SOX 4 in humans compared to animal models of RA (72). Prado *et al*. demonstrated that not all the inflammatory features of RA are directly linked with the severity of the disease, since dendritic cells can lead to the amplification of Th17 and Th1 cells in patients with low grade of disease activity or even in remission phase. They found that the expression of HLA-DR and CD38 as well as the amplification of Th17 response is greater in smoker RA patients than in non-smokers, proving that tobacco can modulate the activity of dendritic cells towards an inflammatory phenotype (73).

As far as B lymphocytes are concerned, in some RA patients, B cells produce anti-PAD4 antibodies, which can increase the action of PAD4 and the consequent production of citrullinated antigens, by lowering the calcium requirement for the enzyme activity. By cloning some of these antibodies from three RA patients Jing Shi *et al*. demonstrated that they are not polyreactive, independently of somatic hypermutations (SHM), and apparently deriving from an *anti-PAD4 B cell precursor.* In order to achieve an effective action against PAD4 they need an affinity maturation and accumulation of somatic mutations the former being facilitated by chronic exposure to active PAD4 (74). In the CIA model, the induction of germinal centres (GCs), B cells and therefore the development of GCs in spleen and in draining lymph node is observed. This is critically linked to the titres of anti-collagen II antibodies and the class switch recombination and consequently to the CIA development. Conversely, mice unable to form GCs or without mature B cell function are protected from CIA if treated with type II collagen, even if reactive T cells can be produced. These animals, however, are fully susceptible to the disease if they are treated with anti-collagen II antibodies, suggesting that targeting the GC reaction could allow for therapeutic interventions that are more refined than general B cell depletion (75). Concerning the role of T lymphocytes on the pathogenesis of bone damage, a recent Korean GWAS highlighted the involvement of $CD8^+$ memory T cells in the development of bone erosions in RA patients. The study revealed that B cells are able to inhibit bone formation in the animal model of RA, by producing multiple inhibitors of osteoblasts, including CCL3 and TNF (76) (77). It is well known that B cells from RA do not adequately respond to DMARDs *in-vitro* when compared to B cells from healthy subjects. This is probably due to a persistent abnormal production of IL8, Gro-α and other pro-inflammatory cytokines and to the deficiency of protective factors such as TRAIL. In addition, Fleischer *et al*. demonstrated the role of IL6 in B-cell derived cytokines in RA, using anti-IL6 approaches. In fact, the addition of tocilizumab in patients who failed previous DMARDs is able to rebalance cytokine production and in the case of MIP-1β and β-NGF by B cells this parallels the clinical response of the therapy. Therefore, it has been suggested that the assessment of B cell cytokine production could help to predict RA patients' response to tocilizumab before starting the treatment (78). In addition, Tsujimura *et al*. have better characterised the expression of P-glycoprotein (P-gp) on activated B cells, the molecule associated with active efflux of intracellular drugs, resulting in drug resistance. According to their results it is possible to hypothesise that the expansion of $P\text{-}gp^+CXCR4^+B$ cells in RA is associated with drug resistance, high grade of disease activity and progressive destructive arthritis with extra-articular involvement (79).

Conclusions

In the last year, several studies have been published in order to better understand the pathogenic mechanisms underlying RA. Several cellular components, soluble mediators, adhesion molecules and autoantibodies contribute to the development of inflammation and structural changes of joints and internal organs. Recently, research at molecular and cellular levels have clarified some mechanisms that regulate the innate and adaptive immune responses in RA. In particular, genetic and epigenetic studies have allowed us to identify new mechanisms that may explain the development of the disease. Research aimed at better clarifying the mechanisms underlying the pathogenesis of RA, will enable the development of novel and more specific disease-modifying therapies.

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