Pathogenesis of rheumatoid arthritis: one year in review 2023

F.M. Mariani[1], I. Martelli[2], F. Pistone[2], E. Chericoni[2], I. Puxeddu[2], A. Alunno[1]

[1]*Department of Life, Health & Environmental Sciences, University of L'Aquila; Internal Medicine and Nephrology Division, ASL 1 Avezzano-Sulmona-L'Aquila, San Salvatore Hospital, L'Aquila, Italy;*
[2]*Immuno-Allergology Unit, Department of Clinical and Experimental Medicine, University of Pisa, Italy.*

Francesco Maria Mariani, MD
Irene Martelli, MD
Francesca Pistone, MD
Erica Chericoni, MD
Ilaria Puxeddu, MD, PhD
Alessia Alunno, MD, PhD

Please address correspondence to:
Alessia Alunno
SSD Reumatologia,
Dipartimento di Medicina Clinica e Sperimentale,
Piazzale Salvatore Tommasi 1,
67100 L'Aquila, Italy.
E-mail: alessia.alunno82@gmail.com

Received on April 11, 2023; accepted in revised form on June 29, 2023.

Clin Exp Rheumatol 2023; 41: 1725-1734.

Key words: rheumatoid arthritis, pathogenesis, innate immunity, adaptive immunity, lymphocytes, cytokines

Competing interests: none declared.

ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by local and systemic inflammation. The complex interplay between immune cells and soluble mediators leads to the induction and perpetuation of aberrant inflammatory and autoimmune responses. The research carried out in the last year in the field of RA enabled the identification of new mechanisms involved in the pathogenesis of the disease and therefore unmasked new potential therapeutic targets. In this review article we summarised the new insights into RA pathogenesis from original research articles published in the last year.

Introduction

Rheumatoid arthritis (RA) is one of the most common systemic autoimmune diseases, and is characterised by synovial inflammation leading to joint destruction and extra-articular manifestations if left untreated (1). In the last year, advances have been made in the knowledge of RA pathogenesis, in particular in the field of genetics and environmental factors that can influence the development of the disease. Due to the technological progress, it has been possible to better characterise cellular and molecular processes involved in the dysregulation of innate and acquired immune responses with the identification of new pathways useful for the development of new therapeutic approaches in RA.

In this review article we summarised the results of a Medline search of original research articles in English published in the PubMed database from January 1 to December 31, 2022.

Genetic and epigenetic advances

The pathogenesis of RA is characterised by the complex interaction of genetic predisposition, epigenetic regulation and environmental factors. Although several mechanisms underlying the disease are not yet completely defined, many advances have been achieved in the last year. Due to multiple genome-wide associations studies (GWAS) and progress in genomic sequencing technologies, several loci linked to an increased risk of RA have been discovered. Among them, both the human leukocyte antigen (HLA) and the non-HLA genes were found to be deeply involved (2).

The HLA region was the first identified locus and the main susceptibility factor associated to RA. In particular, the HLA-II genes are well characterised and the DRB1 was recognised to be crucial for some clinical features of the disease. In fact, the HLA-DRB1*04, HLA-DRB1*01 and HLA-DRB1*10 alleles are associated with an increased risk of RA, and the HLA-DRB1*04:01 also with anti-a501-515cit antibody positivity (2, 3). Furthermore, HLA-DRB1 alleles are associated not only with the severity of the disease and the development of more bone erosions and destruction of joint cartilage (4), but also with a high risk of developing seropositive RA (3). This is the case of anti-cyclic citrullinated peptide antibody (ACPA)-positive patients in whom independent associations hits have been detected in the HLA locus within and outside of HLA-DRB1. On the other hand, the association profile of amino acids from HLA-DRB1 locus resulted widely different in ACPA-negative patients (3). In fact, in this subgroup the highest risk for developing the disease was found for amino acids leucine (Leu) and serine (Ser) at position 11 of HLA- DRB1. We have to take in account that studies on the genetic aspects of RA showed that the HLA association with the disease in European populations differs from other populations like

those of Asian or African ethnicity. In Europeans the association with RA is mainly with HLADRB*0401, HLA-DRB*0404 and HLA-DRB*0101, whereas in the Asian population this association is more specifically related to the HLA-DRB1*04:05 allele, relatively rare in the African and European ones.

Besides HLA variants, several other genetic factors have been extensively investigated in the context of RA, and hundreds of non-HLA risk genetic variants have been uncovered, including protein tyrosine phosphatase non-receptor type 22 (PTPN22), peptidylarginine deiminase type 4 gene (PADI-4), cytotoxic T-lymphocyte antigen 4 (CTLA4), signal transducer and activator of transcription 4 (STAT4) and later on tumour necrosis factor alpha induced protein 3 (TNFAIP3). The majority of genes associated with seropositive disease were identified in the interferon (IFN) alpha/beta and IL-12/23 signalling networks. Furthermore, most sequence variants conferring the largest risk of seropositive RA, pointed to causal genes encoding proteins in the Janus kinase (JAK)/STAT-pathway. This includes a missense variant in the STAT4 gene, conferring 2.27-fold risk, larger than the lead signals at the well-known HLA-DRB1 and PTPN22 loci. In addition, other two missense variants in the tyrosine kinase (TYK)2 gene are recognised to affect the levels of the IFN-alpha/beta receptor 1 (IFNAR1) (1). All together these findings support the usefulness of treating seropositive RA patients with JAK and IL-6R inhibitors as well as CTLA4-Ig, but also introduce the possibility to novel therapeutical approaches targeting JAK/STAT-pathway, including inhibitors of fms related receptor tyrosine kinase 3 (FLT3), TYK2 and IFNAR1 (1). Data regarding non-HLA genetic associations with seronegative disease are less consistent, in fact only three confirmed associations have demonstrated genome-wide significance, close to loci ankyrin repeat domain (ANKRD)55, IFN regulatory factor (IRF)4, and Long Intergenic non-protein coding RNA (LINC)01898 (3).

The key role of inflammation in RA pathogenesis is well defined and genes encoding pro-inflammatory cytokines such as IL-1, IL-6, IL-8, IL-15, IL-17, IL-18, IL-23 and TNF-α were extensively investigated over time (5). Selective single nucleotide polymorphisms (SNPs) in certain pro-inflammatory cytokines are linked to clinical and serological features of the disease, including swollen and tender joints, increased levels of erythrocyte sedimentation rate (ESR), disease activity score on 28 joints (DAS-28) and lasting of morning stiffness (5). Among pro-inflammatory cytokines, IL-23 is one of the main relevant mediators in RA pathogenesis (6). In this context, a recent study conducted in the South Aegean region of Turkey proved that the genotypes of IL-23R with rs11805303(TT), rs10889677(AA), rs1004819(AA), and rs7530511(CT) polymorphisms were detected more often in RA patients than healthy controls (6). Particularly, the AA genotype of IL-23R rs1004819 and the CT genotype of IL-23R rs7530511 were associated with an increased risk of developing RA, while the CC genotype of IL-23R rs11805303, rs10889677 and the TT genotype rs2201841 were associated with the increase of ESR and C reactive protein (CRP). In parallel, patients with CC genotype rs11805303 and GG genotype rs1004819 displayed a more active disease compared to other genotypes (6).

As mentioned above, RA-related genes may affect not only different mechanisms underlying RA pathogenesis, but also several clinical aspects of the disease. In the last year particular attention has been given to autophagy, an active process of removing the inflammatory stimulus and restoring homeostatic balance, and to the autophagy related genes. Some of these genes, including protein phosphatase 1 regulatory subunit (PPP1R)15A, MYC, FOS, cyclin dependent kinase inhibitor (CDKN)1A, epidermal growth factor receptor (EGFR), gamma aminobutyric acid type A receptor associated protein like (GABARAPL)1, BCL2-associated athanogene (BAG)3, forkhead homeobox type O (FOXO)1, FOXO3, and BCL2 Interacting Protein (BNIP)3, resulted up-regulated in RA patients compared to healthy subjects, others, as in the case of regulator of protein G signalling (RGS)19, BCL2 associated X (BAX), Fas associated via death domain (FADD), TNF superfamily member (TNFSF)10, cathepsin (CTS) B, caspase 8 , serpin family A member (SERPINA)1, C-X-C motif chemokine receptor (CXCR)4, apolipoprotein L1, and C-C motif chemokine receptor (CCR)2, were down-regulated (7). In addition, some of these genes were validated to be differentially expressed, and they may serve as valuable prognostic markers of RA.

Tissue remodelling is another relevant mechanism of RA pathogenesis and the main pro-fibrogenic mediator regulating tissue remodelling is TGF-β. This has been studied in depth as an anti-inflammatory mediator, but it is also fundamental in the development and maintenance of fibrosis. TGF-β is tightly regulated by suppressor of SMAD proteins, including SMAD4, the canonical cofactor for TGF-β signalling. It has been hypothesised that SMAD4 rs12456284 and rs10502913 genetic variants may have a potential protective role against RA, while the SMAD2 rs1792666 and SMAD7 rs3736242 variants were associated with a more severe course of the disease in the Caucasian population (8).

Despite all these important results published in this last year, more efforts are required in order to completely elucidate the complex genetic scenario involved in RA pathogenesis. Furthermore, it is worth mentioning the importance of epigenetic regulation in the development of RA and in some clinical features of the disease, including DNA methylation, histone modification, chromosome remodelling and RNA expression, affecting and regulating the function and characteristics of certain genes (9). With regard to DNA methylation, the promoter demethylation of IL-6 and IL-10 genes in a single CpG sequence seems to increase the level of certain cytokines involved in RA pathogenesis, whereas DNA methylation on chromosome 10 seems to promote fibroblast-like synoviocytes (FLS) activation, suggesting its role in tissue remodelling (9). In the context of epigenetic regulations, the endogenous small, single-stranded (ss), non-coding RNAs (ncRNAs) are

leading actors in RA pathogenesis, mainly by modulating the levels of DNA methylation or changing histone modifications, which in turn affect the onset and progression of the disease (9). Numerous micro (mi)RNAs are abnormally expressed in RA-derived cells and are able to regulate target genes and pathways, including NF-κB, Fas-FasL, JAK-STAT, and mammalian target of rapamycin (mTOR) pathways (10). This is the case of miR-21, miR-26a-5p, miR-126, miR-135a, miR-138, miR-143, miR-145, miR-155, and miR-421, FLS-related miRNAs that are mainly over-expressed in RA, whereas miR-19a, miR-20a, miR-27a, miR-29a, miR-34a, miR-137, miR-140-3p, miR-152, and miR-495 resulted down-regulated (11). In the last year, particular attention has been given to miR-22, less expressed in the synovial tissue, and to cysteine-rich angiogenic inducer (Cyr)61, which instead resulted up-regulated, leading to increase proliferation of RA-derived FLS. The circulating levels of miR-22 were increased in rheumatoid factor (RF)-positive, but not in seronegative RA patients, limiting the use of this miRNA as possible disease biomarker (12).

Among ncRNA, long ncRNAs (l) are well known to be abnormally expressed in RA-derived FLS (11). In these cells some specific lncRNA, including lncRNA negative regulator of FLS migration (LERFS), metastasis associated lung adenocarcinoma transcript (MALAT)1, urothelial cancer associated (UCA)1, growth arrest specific (GAS)5 and maternally expressed (MEG)3 are down-regulated, whereas gastric adenocarcinoma associated positive CD44 regulator lncRNA (GAPLINC), Lnc-IL7R, intersectin (ITSN)1-2, plasmocytoma variant translocation (PVT)1, H19, ZNFX1 antisense RNA (ZFAS)1 and p38 inhibited cutaneous squamous cell carcinoma associated lincRNA (PICSAR) are up-regulated. Furthermore, a dysregulation of lncRNAs seems to exert an effect on the functional activities of these cells (11). For example, lincRNA-p21 is down-regulated in RA-derived FLS and contributes to increase their pro-inflammatory functions with consequent amplification and progression of the synovial inflammation (11). Endogenous non-coding RNAs characterised by a closed circular structure (circRNAs) may also be implicated in some aspect of RA pathogenesis. The circ-0088194, up-regulated in RA-derived FLS, promotes the expression of downstream target gene of the metalloproteases (MMP)-2, contributing to the invasion and migration of FLS in the synovia. In addition, the circ-AFF2 increased in the synovial tissue and in the RA-derived FLS, seems to enhance the expression of downstream target 2',3'-cyclic nucleotide phosphodiesterase (CNP), by binding to the miR-650, promoting FLS proliferation, migration and their pro-inflammatory and pro-fibrotic activities (11).

Take home messages

- SNPs of IL-23 receptor modulate the risk to develop RA (6).
- Autophagy-related genes are differentially expressed in RA, outlining the key role of this process in the development of the disease (7).
- Numerous micro (mi)RNAs are abnormally expressed in RA and are able to regulate key pathways involved in disease pathogenesis such as Fas-FasL and JAK-STAT (10-12).

New insights into environmental factors

The important role of environmental factors in RA pathogenesis is well established and among them, smoking has been extensively investigated. Several studies demonstrated that smoking plays a significant role in the disease development, particularly in men who have positive RF and in those who smoke for ≥20 pack-years. On the contrary, smoking cessation may prevent the development of seropositive RA (2, 13). Among the active components of the cigarette smoke, nicotine seems to affect directly the immune system by inducing neutrophil extracellular trap (NET) formation, leading to antigen exposure and consequent ACPA formation (13). Furthermore, smoking seems to promote hypomethylation of certain DNA regions and to increase the levels of pro-inflammatory cytokines, including IL-17 (4). The exposure to maternal smoking during pregnancy has also been investigated and it is able to modify the new born gene methylation pattern, ultimately increasing the risk of developing RA (14).

Besides smoking, other environmental factors have been linked to RA development. For example, some dietary habits seem to exert beneficial effects in preventing the development of the disease. This is the case of healthy diet, which includes omega-3 fatty acids, fish and olive oil consumption, that has some protective effects on the production of autoantibodies associated with RA. On the contrary, high consumption of protein and red meat alongside low intakes of vegetables, olive oil, and vitamins have been related to an increased risk of inflammatory polyarthritis or RA (4). Due to antioxidant, anti-inflammatory and immuno-modulatory properties stemming from the production of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) and polyphenols (15), the Mediterranean diet seems to reduce the severity of some clinical features of the disease. In fact, dietary changes can exert a direct beneficial effect on the function of gut microbiota in RA patients, improving the intestinal barrier and reducing the systemic inflammatory state. In parallel, high salt consumption or sugar sweetened soda may have a negative effect on gut microbiota, increasing the presence of *Prevotella copri* strains with higher proportions of branched chain amino acids (BCAA) and *Lactobacillus* depletion, contributing to increase the intestinal inflammation (16). An increase in *Prevotella copri* in the gut microbiota of RA patients may be responsible for Pc-p27 protein production, which could trigger a Th1-mediated immune response by binding to HLA-DR, promoting activation of the adaptive immune system (15). Conversely, omega-3 intake, the Mediterranean, probiotics and fibre-rich diets are recognised to exert positive effects on gut microbiota (15, 16). Therefore, an appropriate and well-balanced diet should at least be considered as an important adjuvant to medical treatment (16), but it cannot and should not replace the pharmacological management of the disease.

It is well known that microorganisms and viruses can be triggers for RA, acting on the host immune system at different levels: molecular mimicry, epitope spreading, polyclonal lymphocyte activation, bystander activation and viral persistence (14). For example, the presence of *Porphyromonas Gingivalis*, *Aggregatibacter actinomycetemcomitans* or other periodontal bacteria is associated with an increased risk of developing RA, since they are able to interact with the host immune system. In addition, since these are the only microorganisms owing the machinery to citrullinate proteins, they may citrullinate host and bacterial peptides, leading to immune tolerance breakdown and ultimately inducing ACPA formation (4). In addition, different periodontal bacteria species are known to promote RF production by acting on B cell receptor signalling, B cell proliferation, activation and differentiation in RA mouse models. These microorganisms are also able to interfere with $CD4^+$ T lymphocytes co-stimulation and cytokine production (17).

Over the last year, particular attention has been given to SARS-CoV-2 infection. This virus can exert its effect directly on cells in the mouth, lung and gut mucosa, leading to the induction of PADI-4 and in turn to the formation of citrullinated histones. Furthermore, this process seems to become chronic in post-active COVID-19, since lung sensory neurons and the gut represent mucosal reservoirs for SARS-CoV-2 (18). As observed in other autoimmune diseases, also in RA the SARS-CoV-2 infection seems to induce modification of the innate immune response with an initial delayed production of IFN type I, while the NF-kB and inflammasome pathways are activated. Furthermore, in lung and digestive tissues an alternative and extrafollicular immune response against SARS-CoV-2 takes place, leading to an altered humoral and memory T cell responses and ultimately to a breakdown of tolerance and production of autoantibodies (18).

In addition to microorganisms, hormones may affect RA development and some clinical features of the disease, acting directly on the immune system. This is the case of oestrogens, that are able to enhance humoral immunity by stimulating B cell and Th2 response, and promoting autoreactive B and T cell survival, while androgens and progesterone exert an opposite effect by suppressing the immune response (4).

Among the environmental factors, polycyclic aromatic hydrocarbons (PAHs), have attracted increasing attention in the context of RA pathogenesis. These are ubiquitous air pollutants formed by the burning of natural gas, oil that were linked to some mechanisms underlying RA pathogenesis. Overexposure to PAHs usually activates the aryl hydrocarbon receptor (AHR) in immune cells, such as Th1 and Th17 cells, resulting in inflammatory cytokine production and in increased RA incidence (19). Additionally, PAHs inhibit the activation of AHR on Th2 and T regulatory (reg) cells and reduce the ability to produce IL-10, TGF-β and IL-4, that are crucial for maintaining Th1/Th2 balance (19). The key role of PAHs in RA is primarily due to their direct effect on innate and adaptive immune cells, mainly by acting on AHR signalling pathway (19). Interestingly, on Th1, Th17, B cells, dendritic cells (DCs), M1 macrophages and natural killer cells, AHR activation can exert a negative effect whereas on Th2, Treg cells and M2 macrophages a positive one (19). According to previous and recent studies, exposure to any occupational inhalant agents seems to be associated with an increased risk of developing ACPA-positive RA, rising with the numbers and duration of exposures (20).

Finally, socioeconomic status and psychosocial stress are probably linked to some mechanisms involved in RA pathogenesis, but further studies are required in order to define their specific role. If we take in account that eating habits and the socioeconomic status can contribute to the development of obesity, a condition that leads to increase the general inflammatory state of the individual, it is easy to hypothesise a connection between high body mass index (BMI) and the risk of developing RA (13). In fact, in RA obese patient pro-inflammatory cytokines derived from the adipose tissue actively contribute to the amplification of local and systemic inflammation. Therefore, reducing BMI by exercise may lead to decrease the release of inflammatory mediators such as TNF and IL-6, with consequent improvement of some clinical features of the disease (21). Therefore, both aerobic and strength exercise training should be recommended for all patients with RA as part of their routine treatment (21).

Take home messages

- An unhealthy diet may increase inflammation by inducing an imbalance between different strains of gut microbiome (15, 16).
- SARS-CoV-2 infection may induce a long-term abnormal activation of the immune system and ultimately may trigger a breakdown of tolerance (18).
- PAHs are ubiquitous air pollutants that activate the AHR on immune cells, inducing an imbalance towards inflammatory components (19).

Novelties in the innate immune response

The innate immune system, including cells and soluble mediators, plays an active role in the initiation and progression of RA. It is demonstrated that increased activation of the NLR Family Pyrin Domain Containing (NLRP)3 inflammatory complex leads to excessive inflammation with consequent tissue damage and worsening of the disease (22). Up-regulation of expression and activity of NLRP3 in RA patients leads to increase the release of pro-inflammatory cytokines belong to IL-1 family, including IL-1β and IL-18 (22). Therefore, NLRP3 protein expression via activation of NF-κB pathway, is increased in RA-derived monocytes, macrophages, and DC, suggesting a direct contribution of NLRP3 activation in both local and systemic inflammation (22). Furthermore, this activity seems to be tightly regulated by other pathways involved in the mechanisms underlying RA pathogenesis beside the NF-κB. In fact, the activation of NLRP3 inflammasome can be inhibited by autophagy through p-62-dependent clearance of damaged mitochondria (23), and the

activation of autophagy via resveratrol is able to reduce the severity of RA in animal models, limiting the crosstalk among inflammatory cells (23). Autophagy regulates also other mechanisms involved in RA pathogenesis. For example, it exerts a direct effect on the survival of immune cells, on the presentation of citrullinated peptides and on the activation of osteoclasts and FLS (24). In addition, an increased activation of autophagy may lead to apoptotic resistance, increased cell proliferation and production of inflammatory mediators, contributing to further destruction of joints and cartilage (24).

Among IL-1 family cytokines, IL-1β is one of the main pro-inflammatory mediators involved in RA pathogenesis. It is well known its role as suppressor of osteogenic differentiation by dampening the bone morphogenic protein (BMP)/SMAD pathway and of other osteogenic markers such as runt-related transcription factor (RUNX) 2, osteocalcin and alkaline phosphatase (25). This cytokine is also able to promote activation of osteoclasts mainly via a RANKL-RANK independent pathway (25).

Moreover, activation of the FcγR signalling promotes osteoclast differentiation and bone loss in RA, whereas IFN-γ released by immune cells inhibits osteoclast activation. Interestingly, the inhibitory effect of IFN-γ on osteoclast differentiation depends on the stage of this process. In fact, while activation of IFN-γR leads to inhibit osteoclasts formation in early osteoclast precursors, in premature osteoclasts this activation exerts an opposite effect (26).

Besides the IL-1 family cytokines, in the recent year great attention has been given to IL-34. This is a cytokine discovered a few years ago and identified as the second colony-stimulating factor (CSF)-1R ligand, involved in homeostasis as well as pathogenetic processes. Recently, the role of this cytokine in RA pathogenesis has been investigated and has been shown that *in-vitro* stimulation of RA-derived FLS by IL-34 modulates selectively FLS activity by inducing the synthesis of IL-8 and TNF-α, but not of IL-6, and this effect could be impaired by signal inhibitors (27). Furthermore, IL-34 can modulate the production of downstream inflammatory mediators via multiple signalling pathways, suggesting its contribution to the progression of the disease (27).

Among the innate immune cells involved in RA pathogenesis, DC are recognised to be key cells in the initiation and progression of the inflammatory processes. In this context, a novel CD209/CD14$^+$ DC population differentiated by monocytes has been recently identified in highly frequency in the circulation of patients with RA and psoriatic arthritic (PsA) (28). They also express more pro-inflammatory cytokines IL-1β, IL-6, IL-12 and TNF-α and are increased in the inflamed joint where they display a unique inflammatory phenotype with high expression of CD40, CD80 and chemokine receptors (28). Furthermore, following activation by TNF-α via JAK/STAT pathway these cells increase the synthesis and release of pro-inflammatory cytokines. In parallel, analysis of specific myeloid subsets including CD1c$^+$ conventional (c) DC showed an increase in the circulating of CD1c$^+$ DC with high CCR2 and IgG receptor CD64 expression, and the same DC phenotype was also increased in the synovia. Moreover, studies *in-vitro* showed that synovial CD1c$^+$ cDC exert a pro-inflammatory activity and have high ability to induce pathogenic IFN-γ$^+$IL-17$^+$CD4$^+$ T cells (29).

Besides the synovial DC, a key role in RA pathogenesis is played by macrophages exerting their activity by releasing TNF-α, IL-1β, IL-6, CCL2, IL-8, MMP-3 and MMP-12. Different macrophage phenotypes were recently associated with different disease activity. While in active disease M1 macrophages are prevalent in both peripheral blood and synovial tissue, higher proportions of M2 macrophages with a phagocytic activity were identified in the remission stage of the disease (30).

It is worth mentioning that the innate and the adaptive immune systems are tightly linked in initiation and progression of RA. This has been recently confirmed by analysis of sputum neutrophils obtained from subjects at risk of developing RA and in RA patients resulted more prone to spontaneously undergo NET formation. Interestingly, in those at-risk to develop RA the level of IgA ACPA in the lung was associated with increased Cit-H3–expressing NET formation and strongly correlated with Cit-H3– containing NET remnants increased in the sputum of these patients, containing higher levels of the pro-inflammatory cytokines IL-1, IL-6, and TNF (31). The active role of NETs in RA pathogenesis has been widely studied in the recent year. NETs can be directly involved in the inflammatory response by exposing pro-inflammatory mediators, and in the auto-antibodies productions by exposing autoantigens (32). Due to their role in protein citrullination, NETs might induce ACPA formation and in some cases might be targets for these autoantibodies. In both cases, NETs may form immune complexes (ICs) and trigger downstream pathogenic mechanisms (33).

Among innate immune cells recent attention has been given to the innate-like T cells, including NKT and mucosal-associated innate T (MAIT) cells. These are specialised T cells with innate characteristics that express semi-invariant T cell receptors with non-peptide antigen specificity. In particular, the MAIT cells are able to recognise antigens that are presented by the monomorphic MHC-I related molecule (MR)1. In addition, MAIT cells express the homing receptors CCR5, CCR6 and CCR9, and the transcription factors T-bet, retinoic-acid-receptor-related orphan nuclear receptor gamma (RORγt), and promyelocytic leukaemia zinc finger protein (PLZF). MAIT cells can also be activated in a T-cell receptor (TCR)-dependent manner and by inflammatory cytokines like IL-12, IL-18 and IL-7 (34). In response, MAIT cells secret Th1 and Th17 type cytokines and release granzyme B and perforin. Recently it has been observed that RA-derived synovial fluid (SF) has a higher percentage of MAIT cells and in the early stage of the disease the distribution of these cells shifts from CD8$^+$ to CD4$^+$ MAIT cells with expression of low levels of CD161 (34). In addition, the role of these cells in RA pathogenesis has been demonstrated in a MAIT cell-deficient MR1-/- mouse model of arthritis, where the severity of collagen-

induced arthritis (CIA) was ameliorated (34). Furthermore, a pro-inflammatory environment might actively modulate MAIT cells. In fact, treatment of these cells with IL-1β leads to increase the cell proliferation, while treatment with IL-23 leads to produce IL-17 in the absence of TCR stimulation, suggesting a direct contribution of MAIT cells in the amplification and perpetuation of the inflammatory processes (34).

Take home messages

- Autophagy regulates several mechanisms involved in RA pathogenesis including the NLRP3 inflammasome pathway (22, 23).
- A novel CD209/CD14$^+$ DC population with a unique inflammatory phenotype has been identified and these cells are expanded in RA joints (28).
- MAIT cells are specialised T cells with innate characteristics that are involved in the amplification and perpetuation of the inflammatory processes in RA joints (34).

New findings in adaptative immunity

Most of the studies on the role of T cells in the pathogenesis of RA have primarily focused on the characterisation and function of Th1, Th2, Th17, and Treg cells from the peripheral blood and inflamed joints during established disease.

It is now widely recognised that several factors might be responsible for the imbalance between Th and Treg cells in RA. Among them, STIP1-homologous U-Box containing protein 1 (STUB1), miRNA-146a, IL-3 and DJ-1 have been widely investigated. In particular, STUB1 promotes AHR non-degradative ubiquitination, inducing the development of a Th17 phenotype. In addition, the high STUB1 level detected in the circulation of RA patients was probably due to a selective increased expression of this protein in Th17 cells. Following modulation of STUB1 expression in CD4$^+$ sorted cells, different levels of STUB1 were able to induced changes in expression of the transcription factors RORγt and forkhead box protein 3 (FoxP3) as well as in the soluble mediators produced by Treg and Th17 cells. These important results suggest that if an upregulation of STUB1 promotes Th17 cell differentiation and suppresses Treg differentiation, its down-regulation is able to exert the opposite effect, supporting the key role of STUB1 in Th17/Treg balance (35).

The link between mi-RNA 146a and RA has been already recognised and several studies showed its differential expression in various immune and non-immune cells derived from RA patients (36). Increased expression of circulating miR-146a has been confirmed in a population of elderly RA patients, supporting the link between the levels of miRNA-146a and the balance of Th17/Treg cells. In fact, the levels of miRNA-146a correlate directly with the mRNA levels of the retinoic acid-related orphan receptor variant 2 (RORc) as well as with the percentage of circulating Th17 cells and IL-17 levels. On the contrary, they inversely correlate with mRNA levels of FoxP3, percentage of circulating Treg cells and TGF- β1 levels (37).

In parallel, particular attention has been given to IL-3, a cytokine secreted by basophils and activated T cells that supports the growth and differentiation of mucosal mast cells and T cells. Rani *et al*. shed some light on the possible involvement of this cytokine in the pathogenesis of RA by demonstrating that IL-3 inhibits the differentiation of both mouse and human Th17 cells. In addition, this cytokine promotes the development of Treg in IL-2-dependent manner, by modulating the expression and phosphorylation of the STAT components. In fact, it has been proved that IL-3 was able to up-regulate STAT2, STAT5, and STAT6, known to inhibit Th17 development, and to downregulate STAT1, STAT3, STAT4, and NOS2 genes, responsible for inducing Th17 cells differentiation and activation. This inhibitory effect was further supported by exogenous administration of IL-3 in CIA model, leading to a significant reduction of the articular inflammatory infiltrate (38).

Regarding DJ-1, initially identified in Parkinson's disease, a previous study has demonstrated that DJ-1-knockout mice showed higher arthritis scores and incidence rates than those of wild-type mice (39). Furthermore, higher levels of DJ-1 were detected in the serum and synovial fluid of patients with RA, but not in those with osteoarthritis, and *in-vitro* it was able to inhibit RA-FLS activities, including inhibition of RANK-L dependent osteoclastogenesis and decrease of vascular endothelial growth factor (VEGF) and TNF-α.

In parallel, cell division control protein (CDC)42, a small GTPase belonging to the Rho family, able to modulate the immune response by interacting with neutrophils, T and B cells, has been investigated. CDC42 hampers Th17 cell differentiation thereby influencing the Treg/Th17 balance, while its silencing promotes Th1 cell differentiation. CDC42 serum levels were inversely correlated to the proportion of circulating Th17 cells, ESR, CRP, and DAS28, but they were not linked to Th1 cells, ACPA and RF positivity. Although intriguing, these data need to be further confirmed in larger studies also assessing in more detail the potential link between CDC42 and the cellular counterpart (40). As demonstrated in the regulation of the innate immune system of RA, MALT-1 is also involved in adaptative immunity. MALT-1 is able to activate the NF-κB pathway and has a substantial impact on the development of Th17 and Th1 cells. Although research studies proved that MALT-1 correlates with circulating Th17 cells, conflicting data were obtained regarding Th1 cells (41).

As mentioned above, circRNAs are important transcriptional regulators of genome expression. Among them, hsa_circ_0089172 (circNUP214) has been demonstrated for the first time in RA and transcript levels of circNUP214 were directly correlated with those of IL23R and with circulating Th17 cells. In addition, by circNUP214 knockdown/down-regulation, it was possible to reduce the Th17 cell component alongside the transcript levels of IL-17A (42).

In the last year particular attention has also been given to the modulatory role of TNF-α on Th1 and Th17 cells. In fact, TNF-α was proved to decrease the number of IFN-γ-producing T cells, including both Th1 and Th17 cells, as

well as to increase IL-17 production, suggesting that TNF-α exerts an effect on maintaining the Th17 phenotype and on promoting the persistence of Th17 cells (43).

In parallel to the insights on Th1 and Th17 cells in RA, intriguing results were obtained on characterisation of Treg cells. In this regard, the proportion of $CD4^+CD25^+Foxp3^+$ Treg cells and memory Treg cells were higher in the SF of RA then in the peripheral blood of these patients and healthy subjects, resulting functionally defective and expressing lower levels of CD73 and TGF-β1 (44).

A new $CD4^+FoxP3^+$ Treg cell subset lacking CD25 has also been identified in RA. These cells express low levels of Helios, a transcription factor responsible for the maintenance of a suppressive and anergic phenotype of Treg, and high levels of IFN-γ, suggesting a potential dual role of these cells as anti and pro-inflammatory (45).

In the complex scenario of the adaptive immune system, osteopontin (OPN) a molecule involved in preventing apoptosis and tissue repair, is now recognised to play an active role and important function in the perpetuation of chronic inflammation in RA. In fact, OPN deficiency prevents joint swelling and destruction as well as cartilage proteoglycan loss in anti-type II collagen antibody-induced arthritis in mice (46). In humans, this mediator resulted higher in patients with active RA versus non active disease and paralleled the lowest proportions of Treg cells, suggesting its potential direct role in the dysregulation of Treg/T effector cells balance (47).

In this scenario, it has been recently showed that Poly(rC)-binding protein (PCBP)1, an iron chaperon protein involved in post-transcriptional regulation, might be involved in the maintenance of the balance between Treg and T effector cells. PCBP1 was found decreased in RA Th1 cells, and by silencing PCBP1 it is possible to exert an effect not only on the transcription of various proteins involved in the inflammatory response, but also on the levels and variety of alternative splicing in Th1 cells. Even if these results are potentially relevant in RA pathogenesis, additional studies are required in order to clarify the precise role of PCBP1 in the disease (48).

It is well known that the amplification of inflammation and its perpetuation might be in part due to some changes in the cellular metabolism of the adaptive immune system. In $CD4^+$ T cells from RA patients the tricarboxylic acid TCA cycle and fatty acid oxidation are deeply defective, leading to the accumulation of lipid droplets and the formation of large membrane structures, promoting tissue invasion. In parallel, in untreated RA $CD8^+$ T cells, but not in healthy control, an elevated rate of aerobic glycolysis, lactate production as well as high levels of ROS were detected (49).

The dysregulation of different T cell subsets in RA pathogenesis was investigated at different levels. For example, hyperactivity of $CD8^+$ T cells in RA has been previously demonstrated and at least in part attributed to Toll-like receptor (TLR)4-dependent activation. However, recently it has been showed that other TLRs might be involved. This is the case of TLR7 increased in the circulating $CD8^+$ T cells of RA patients and demonstrated to contribute to the perpetuation of inflammatory processes (50).

The cytotoxic activity of $CD8^+$ T cells is partially due to their mediators. Recent single-cell transcriptomics and mass cytometry analyses of RA-derived synovial tissue revealed that $CD8^+$ T cells can be divided into the following clusters: granzyme K+ granulysin+ granzyme B and IFNγ-producing granzyme K + granzyme B cells. To better understand how these lymphocytes are involved in RA pathogenesis and find new disease markers, Zhang *et al*. focused on $CD8^+$ cells immunometabolism and in particular on the possible role in RA of mTOR, a strong enhancer of glycolysis. Interestingly, the levels of the phosphorylated (p) form of mTOR resulted higher in RA $CD8^+$ cells compared to healthy $CD8^+$ cells. Furthermore, p-mTOR levels positively correlate with disease activity and, following *in-vitro* stimulation, these cells were able to produce granzyme B, granulysin, TNF-α and IFN in parallel to the decrease production of granzyme K. Interestingly, following treatment with TNF-α inhibitors, the levels of p-mTOR levels decreased, suggesting that mTOR activation could be a potential therapeutic target in RA (51).

Research activities in the characterisation of different aspects of the adaptive immune system in RA highlighted several knowledge on T cell subsets. This is the case of T cells co-expressing both CD4 and CD8 surface markers. These double positive T cells seem to derive from CD8 lymphocytes subsequently acquiring $CD4^+$ expression. Since this cell population has been already observed in the circulation of RA patients, Nguyen *et al*. explored these cells in more detail in a large cohort or RA patients, demonstrating that almost 30% of them displayed an expansion of double positive T cells associated with a more severe disease and higher frequency of erosions (52).

Other important T cell subsets such as PD-1-expressing T cells and follicular (f) Th cells were identified in early RA, preclinical RA and in the subjects at-risk to develop the disease. Previous studies on Tfh cells were focused on peripheral blood or inflamed tissue samples, but for the first time, Tfh cells were investigated in secondary lymphoid organs during the early stages of autoimmunity. It has also been hypothesised that Tfh cells may contribute to autoantibody production in the preclinical early stages of the disease by guiding B cell differentiation in secondary lymphoid organs. For the first time increased frequencies of $CD4^+$ and $CD8^+$ Tfh cells were proved to be increased in the lymphoid tissue of patients with early disease and at risk of developing RA, and a link between Tfh and B cell components was showed in lymphoid tissue as well as in synovial tissue of RA patients (53).

We also have to take into account that PD-1-expressing peripheral Th (Tph) cells might contribute to RA development by secreting IL-21 and providing co-stimulatory signals to B cells in germinal centres. However, it is not known at which stage Tph cells accumulate in the synovial tissue of treatment-na-

ive early RA patients. To address this, Murray-Brown and coworkers compared early RA patients to those with osteoarthritis to determine the timing and modality of Tph cells infiltration of the synovium (54). By flow cytometry analysis accumulation of Tph cells, rather than Tfh cells, was showed in the synovial tissue of patients with treatment-naive early RA. In addition, PD-1^{high} Tph cells were more abundant in RA compared to osteoarthritis, while the PD-1^{int} Tph cells were present at comparable levels in the synovia of both diseases, improving the previous data showing a dominant presence of $CD4^+PD\text{-}1^+CXCR5^-$ Tph cells over Tfh cells in the circulation and synovia of RA (55). Further analysis of other surface molecules such as T cell immunoreceptor with Ig and ITIM domains (TIGIT), an immune checkpoint receptor primarily expressed by Treg cells, activated T cells, B cells and NK cells, showed that TIGIT expressing T cells were increased in RA, but also in seropositive compared to the seronegative ones. These results support the hypothesis that individuals with detectable RA autoantibodies, but no clinically evident disease, might represent an intermediate phenotype, sharing some immunological features with patients with overt RA, particularly an increased expression of the immune checkpoint receptor TIGIT, primarily among $CD4^+PD\text{-}1^{high}$ cells (56).

In parallel, it has been showed that the CD4 T-cell polyfunctionality in the synovial tissue precedes the clinical onset of RA, being detectable also in at-risk individuals, supporting the hypothesis of an immunomodulatory polyfunctionality in the synovial tissue of healthy subjects (57).

Besides T cells, the regulation of the adaptive immune system is tightly link to B cells known to have a dual role in the development of RA. These cells, besides generating autoantibodies such as RF and ACPA, and exerting other effector functions, can also counteract inflammation. In fact, B cells can act as antigen-presenting cells (APCs) and produce cytokines that can suppress inflammation, leading to down-regulate growth and differentiation of T cells and to activate macrophages. Surface expression of B cell receptors (BCRs) is mainly responsible for antigen recognition as well as B-cell activation and differentiation into plasma cells. Recent studies focused their attention on the abnormal activation and hyperactivity of B cells in RA, demonstrating that a common TCR signature can be found in the RA synovial tissue. Based on this, it has been evaluated which B-cell clones dominate the BCR repertoire in different biological samples of RA, and it was found that there is a higher concordance between BCRs in different affected joints compared to other compartments. Therefore, BCR clonal responses may be localised to the target tissue of RA, suggesting that therapeutic strategies targeting B cell clones may be worth further investigation as an alternative to broad and less specific immunomodulatory strategies (58).

The knowledge on the role of B cells and adaptive immune response in RA was deeply improved in the last year. For example, extracellular vesicle (EVs), particularly those forming immunocomplexes (lEV-ICs), are considered a source of autoantigens that increase pro-inflammatory responses from innate immune cells. Given that patients with RA have higher levels of these vesicles in their plasma compared to healthy controls, the impact of medium/large size extracellular vesicles (m/lEVs) on B cell function was widely investigated in the contest of RA. By reproducing *in-vitro* the possible lymphocyte dynamics using samples from healthy donors and patients with RA, it was observed that m/lEVs reduced the expression of activation markers, decreased calcium mobilation, and lowered tyrosine phosphorylation of *in-vitro*-activated B cells. Therefore, in the last year research tried to clarify the mechanisms underlying m/lEV effect on B lymphocytes, using autologous monocyte-derived macrophages (MDM) pre-incubated with m/lEVs and then co-cultured. Following this treatment, these cells released higher levels of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α compared to the control cells, and it has been proved that m/lEVs have both direct and indirect effects on B cell activation, contributing to RA pathogenesis (59).

In this complex scenario, inducible co-stimulator (ICOS) and its ligand (ICOSL) seems to be critical regulators of the autoimmune response. ICOS is a member of the CD28 family and is predominantly expressed on T cells, while ICOSL is constitutively induced on various cells such as B cells, macrophages, and DC. Furthermore, the signalling through ICOS/ICOSL is crucial for the interaction between T and B cells, and higher levels of ICOSL in $CD19^+$ B cells was detected in RA compared to osteoarthritis patients and healthy controls. This effect paralleled the increased ICOS in RA $CD4^+$ T cells and the proportion of $CD19^+ICOSL^+$ B-cells was positively correlated with disease activity. Thus, these results were validated in animal models, where the adoptive transfer of $CD19^+ICOSL^+$ B cells worsened arthritis scores, whereas ICOSL blockade improved the disease in CIA mice (60).

The role of B cells in RA is complex and these cells were widely investigated at different levels. This is the case of the recently identified B cell subset, Age-associated B cells (ABCs), demonstrated to be increased in the peripheral blood and in the SF of RA. Furthermore, these cells have distinct transcriptomic properties that may impact their ability to migrate into the inflammatory joints of RA. In addition, co-culturing RA FLS with RA ABC, the latter cells were able to activate FLS and to increase their production of IL-6, MMP-1, MMP-3 and MMP-13 via TNF-α-mediated ERK1/2 and JAK-STAT1 pathways. Finally, by using the CIA model it was demonstrated that murine B cells are able to generate *in-vitro* ABCs after exposure to IL-21 and TLR7 signalling (61).

Parallel to ABCs, the role of regulatory B cells (Breg) in immunosuppressive responses has been documented by several studies. Abnormalities of Breg cells, also known as $B10^+$ cells, producing the immunosuppressive cytokine IL-10, have been described in several autoimmune diseases, chronic infections, cancer, and in transplant rejection based on studies on experimental

disease models and studies in humans (62). A reduced number of circulating Breg cells has been observed in the peripheral blood of RA patients and expression of CXCR5 was higher in Breg cell surface compared with other B cells. Furthermore, its ligand CXCL13 was able to preferentially attract Breg cells and also to increase the production of IL-10. However, the amount of the CXCR5 expression was lower in Breg from RA compared to those from normal subjects, and subsequent studies showed a dysregulation of the CXCL13-CXCR5 axis in this disease (63).

Take home messages

- DJ-1 and IL-3 play protective roles in RA by inducing the commitment of Treg cells and by inhibiting Th17 proliferation, while STUB1 and MALT1 exert pro-inflammatory activities, by promoting Th17 differentiation (35).
- CD4 T-cell polyfunctionality might be active since early phases of RA in the synovial tissue. Increased frequencies of TIGIT-T cells in the blood, Tfh and B cells in the synovial tissue as well as CD4+ and CD8+ Tfh cells in the lymphoid tissue have been detected (56).
- Newly identified age-associated B cells (ABCs) activate FLS through TNF-α-mediated pathways and have distinct transcriptomic properties affecting their ability to migrate into inflammatory joints (61).

Conclusions

Over the last year several research studies have been published shedding additional light on the pathogenic mechanisms underlying RA. In particular, studies on gene susceptibility, epigenetic mechanisms, different environmental factors allowed us to unmask new pathways or better clarify known mechanisms leading to RA development. In parallel, interesting and promising results for future therapeutic developments have been reported in the context of the innate and adaptive immune systems with particular attention to novel lymphocyte and DC subpopulations regulating the pro/anti-inflammatory balance in RA synovial tissue.

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