Review

One year in review 2018: pathogenesis of rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that primarily affects joints. The several mechanisms involved in the development of the disease are not completely understood. It has been proposed that different environmental factors, such as cigarette smoking, occupational and atmospheric agents act as trigger stimuli for the development of RA in genetically predisposed individuals, leading to synovial hyperplasia and bone destruction. The initial disease stage of RA is associated with alteration of innate and adaptive immune system with consequent production of autoantibodies, targeting various molecules including modified self-epitopes. In the following stages of the disease, both the innate (e.g. dendritic cells, macrophages and neutrophils) and adaptive immune cells (e.g. B and T lymphocytes) contribute to the amplification and perpetuation of the chronic inflammatory state. The recognition of key cells, mediators and mechanisms implicated in the pathogenesis of RA could provide the basis for the development of new and precise disease-modifying anti-rheumatic drugs.
Therefore, we reviewed the literature of the last year in order to find the new insights in RA pathogenesis.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory autoimmune disorder characterised by a persistent joint inflammation leading to cartilage and bone damage, disability and eventually to systemic complications. The progression of the disease may lead to lose functionality, reduce quality of life and enhance morbidity and mortality.

RA pathogenesis is the result of a complex interaction between genetic and environmental factors, inducing the aberrant activation of innate and adaptative immune system which cause the breakdown of immune tolerance, autoantigen presentation with antigen-specific T and B cells activation and aberrant inflammatory cytokines production. The cascade of events leads to synovitis, proliferation of synovia and cartilage and subchondral bone destruction. RA can also involve extra-articular organs, mainly skin, lung, eyes and cardiovascular system. The better understanding of pathogenetic pathways underlying RA, may be relevant to obtain more targeted and safer therapies, to improve diagnosis in the early stage of the disease with consequent better disease control.

The aim of this review is to provide an overview of the new insights in RA pathogenesis, summarising the most relevant studies published over the last year.

Genetic aspects

Congenital predisposition is a well known risk factor for RA development. Several studies are focusing on identifying new genetic clues that can be involved in the pathogenetic processes, leading to the development of RA.

A subgroup of the non-canonical Wnt molecule, named Wnt5a, has been recently identified. This molecule is able to modulate cellular differentiation, migration and inflammation. In particular, Wnt5a is up-regulated in fibroblast-like synoviocytes (FLS) of RA patients and it has been implicated as a possible player of arthritis. MacLauchlan *et al.* described for the first time a role for endogenous Wnt5a in autoimmune disease. They studied two population of Tamoxifen-inducible mice, Wnt5a knockout (Wnt5a cKO) and littermate controls, by monitoring for arthritis development and joint pathology. They discovered that Wnt5a cKO mice were resistant to arthritis development, and some parameters of inflammation were reduced, including the extent of cells

infiltration, extra-articular inflammation, cartilage destruction and osteoclast activity. They results suggest that Wnt5a may play a role in the development of arthritis, by promoting inflammation and osteoclast fusion (1).

It is well known that matrix metalloproteinases (MMPs) are the key enzymes responsible for the joint destruction and their activity is highly regulated by proinflammatory cytokines. Recently, Sotjanovic *et al*. investigated the impact of Tumor necrosis factor (TNF)-α G-308A polymorphism on MMP-9 levels in blood plasma (BP) and synovial fluid (SF) of patients with RA, focusing on their role in the progression of joint destruction. They were able to reveal that MMP-9 activity in BP and SF was significantly higher in RA compared to controls, as well as in SF of patients with erosive compared to non-erosive RA. In addition, the presence of TNF-α-308A allele was found to be associated with increased MMP-9 activity in SF from patients with early RA and it may be a predictor of rapid radiographic progression of the disease (2).

Several candidate gene variants have been identified by genetic mapping in RA such as VAV1 polymorphism. VAV1 is a member of a tripartite family of guanine nucleotide exchange factors (GEF) for Rho/Rac GTPases, responsible for bridging extracellular signals into a number of outcomes, ranging from tissue remodelling, cell migration, activation and gene expression. This seems to be present exclusively in haematopoietic cells, acting on the downstream pathway of immune receptors. Guerreiro-Cacais *et al*. showed in rat model of T-cell-dependent pristane-induced arthritis (PIA) a correlation between VAV1 polimorphism expression and the activity of anti-CCP negative RA. Subsequently, these results were confirmed by meta-analysis in case-control studies performed in RA patients from several Caucasian populations, suggesting a contribution of VAV1 gene in anti-CCP negative RA (3).

Furthermore, in an Indian population of RA patients, Mariaselvam *et al*. investigated the influence of polymorphism of NKG2D receptor on predisposition to and modification of the disease phenotype. NKG2D is a C-type lectin receptor present on natural killer (NK) cells, γδ, CD81 and CD41 T cells. Upon ligand binding, NKG2D mediates activatory and co-stimulatory signals to NK cells and activated CD41 T cells, respectively. Their study revealed that A allele of NKG2D9 and T allele of NKG2D10 was significantly higher in patients with deformities, while haplotype analysis revealed that the frequency of haplotype GC-A-G-A-T-C-C was higher in RA patients than in controls, suggesting that the NKG2D gene polymorphisms may modify the risk of development and severity of the disease (4).

Moreover, in order to describe new candidate genes for the development of RA, Shchetynsky *et al*. performed RNA-sequencing-(RNA-seq)-based expression analysis of 377 genes with previously verified RA-associated loci in blood cells from subgroups of RA patients. From this analysis they demonstrated that differences in the expression of ERBB2, TP53 and THOP1 were similar in both treated and non-treated patients with RA, suggesting that ERBB2, TP53 and THOP1 may represent new candidate genes involved in the pathogenesis of the disease (5).

For the same purpose, a gene-based association testing with GATES (Gene-based Association Test using Extended Simes procedure) was performed in 14,361 RA subjects and 43,923 controls of European ancestry, using 8,694,488 single-nucleotide polymorphism (SNPs). They observed that 115 genes were significantly associated with RA by gene-based association testing, corresponding to 43 RA risk loci and particularly 6 new top gene hits for each of the following 6 RA risk loci: RPP14 (for DNASE1L3-ABHD6-PXK), PXT1 (for ETV7), MIR5708 (for TPD52), DDX6 (for CXCR5), SUOX (for CDK2), and PCAT29 (for LOC145837). A new potential RA risk locus (11q23.3, start position 118528941 bp) which contains the following 3 genes: TREH-PHLDB1-MIR6716 was also identified, confirming prior RA risk loci and identifying novel risk genes including non-coding regulatory miRNAs in RA (6).

Furthermore, Kurowska-Stolarska *et al*. provided evidence that MicroRNA (miR)-34a supplies homoeostatic control of CD1c+ dendritic cells (DCs) activation via regulation of tyrosine kinase receptor AXL, an important inhibitory DC auto-regulator. They found that this pathway is aberrant in $CD1c^+$ DCs from patients with RA, with up-regulation of miR-34a and lower levels of AXL compared to DC from healthy donors. In addition, silencing miR-34a in animal model allowed to reduce the production of pro-inflammatory cytokines, while miR-34a-deficient mice were resistant to collagen-induced arthritis (CIA), interfering with the interaction of DCs and T cells (7).

In addition to TNF-α, IL-1b and IL-6 other cytokines such as IL-23, IL-17 and interferon gamma (IFN-γ) also play crucial roles in the pathogenesis of RA. A recent meta-analysis described that IL-17 levels were significantly higher in the RA than in the control groups and that expression of IL-17A rs2275913, IL-17F rs763780 and IL-17A rs3819024 polymorphisms were significantly more expressed in RA patients (8). By selecting an Irish population, McCarthy *et al*. studied the prevalence of α1 -antitrypsin deficiency (AATD) in RA, showing that there were no differences in the prevalence of heterozygous AATD between RA and healthy groups. On the contrary they observed a positive association between heterozygosity for AATD and the production of anti-citrullinated peptide autoantibodies (ACPA) with increased autoantibody titers, assuming that AATD may define a distinct subset of patients with increased disease severity (9).

It is well known that DNA methylation is an epigenetic modification relevant in RA pathogenesis. Rhead *et al*. demonstrated that hypermethylation was present in RA PB compared to controls, supporting that FLS-representative DNA methylation signatures derived from the PB may prove to be valuable biomarkers for the risk of RA or for disease status (10).

Some interesting results on novel genetic factors regulating organ involvement in RA have been recently

published. Particularly Oka *et al*. described the expression of different miR profiles in two Japanase populations of RA with or without Interstitial Lung Disease (ILD). These are small non-coding RNAs with approximate 22 nucleotide length and are stably detected in plasma or serum. It is widely known that they are able to modulate the expression of protein-coding genes at the post-transcription level and play important roles in cell activation, proliferation, differentiation or death. The authors found that expression levels of hsa-miR-214-5p and hsa-miR-7-5p were increased in RA with ILD, identifying for the first time a correlation between miRs and ILD in RA (11).

Enviromental factors

Many environmental factors, such as cigarette smoking (CS), occupational and atmospheric agents, have been proposed as trigger stimuli for the development of RA in genetically predisposed individuals.

Cigarette smoking

Among all the environmental factors that seem to be involved in the pathogenesis of RA, CS in the one with strongest evidence, predisposing to the generation of citrullinated proteins especially in subjects carrying determined SE alleles.

Epidemiological studies have been underlining the association between CS and RA. Recently Svendsen *et al*. confirmed in an historical Danish twin cohort that risk of developing RA is more than doubled after 20 years of smoking in both sexes (12).

New insights about molecular mechanisms and the role of nicotine in the immune process are now available. Meng *et al*. identified a gene-environment interaction between smoking and SNPs in the rs6933349 gene influencing the DNA methylation level of cg21325723. They found that among current smokers, minor allele (rs6933349_A) carriers had a lower level of methylation at cg21325723 which seems to be associated with increased risk of developing ACPA-positive RA (13).

Lee *et al*. found that exposure to high systemic level of nicotine exacerbates the severity of arthritis in the murine model of CIA and induces a dose-dependent NETosis in isolated human neutrophils (14).

Survivin is an inhibitor of apoptosis that prevents the activation of caspases and a regulator of cell cycle progression by aiding formation of the chromosomal passenger complex. Survivin seems to be involved as a mediator of smoking in the pathogenesis of RA as its levels are higher in the sera of RA smoking patients compared to non-smokers and in bone marrow (BM) of CIA mice treated with nicotine. A further analysis of these mice BM showed that nicotine exposure causes $CD8^+$ T cells to adopt a non-exhausted phenotype, characterised by loss of expression of the programmed cell death -1 (PD-1) receptor and induction of IL-7 receptor, associated with loss of tolerance and development of arthritis (15). Furthermore, Andersson *et al*. found that smoking is associated with the expression of all the three isoforms of survivin in the sera of RA patients and with deregulation of miR processing machinery. The latter results in a restricted global expression of miR through the reduction of Dicer1 endonuclease levels, necessary for the cleavage of the system loop structure of pre-miR. Increased levels of Dicer1 and restoration of miR production was achieved by treating human leukocytes with non-selective inhibition of all the three survivin isoforms (16).

Occupational and atmospheric agents

Recently, some evidences indicate a possible correlation between the development of RA and the exposure to occupational and atmospheric agents. High risk of developing ACPA-positive RA was observed among silica-exposed smokers in a small Swedish cohort (17). Furthermore, Bernatsky *et al*. provided evidence that industrial air pollution emissions and proximity to major industrial emitters are associated with positivity for ACPA in the Canadian CARaGENE cohort (18). In addition, it has been suggested that the exposure to some pesticides may play a role in the development of RA among male farmers. Meyer *et al*. found a dose-response association between RA development and atrazine, a commonly used herbicide, as well as toxaphene, an organochlorine insecticide, regardless of age, smoking and educational level (19). Ilar *et al*. found an increased risk of developing RA in electronics workers, bricklayers, concrete workers, material handling operators among men and assistant nurse and attendants among women in the Swedish EIRA cohort (20).

Diet

Among the individual components of the Alternative Healthy Eating Index (AHEI-2010), a dietary quality score based on the Dietary Guidelines for Americans, both moderate alcohol consumption and lower red meat intake were found to be most associated with decreased-onset RA risk (21). In addition, low sodium intake in RA patients seems to reduce the expression of IL-9 and transforming growth factor (TGF)-β, suggesting that a restricted sodium dietary intake could contribute to dampen the pro-inflammatory response (22). In this regard, also short-term low magnesium intake seems to have protective effect on arthritis severity in a murine model of arthritis (23).

Microbiota and infections

A growing number of studies underscores the association between the development of RA and periodontitis. In this setting *Porphyromonas Gingivalis* infection is of particular importance and represents the link between periodontitis and citrullination since P. gingivalis is the only bacterium constitutively equipped with the peptidyl arginine deiminase (PAD) enzyme. Schmikler *et al*. confirmed an association between development of RA and worse oral health conditions, (24) and the presence of *P. Gingivalis* in periodontal pocket seems associated to RA autoantibodies (25).

Oral and intraperitoneal inoculation of *Porphyromonas Gingivalis* in the CIA murine model (especially after the immunisation process) seems to increase synovial inflammation and expression of several synovial protein like enolase and fibronectin and their citrullinated forms (26).

An analysis of the citrullinome of gingival crevicular fluid (GCF) and periodontal tissue, identified an overlap between periodontal citrullinated peptides and citrullinated peptides recognised by autoantibodies in RA. A novel peptide was recognised as target of autoantibodies, the cytokeratin 13 (cCK13-1) which has a direct correlation with anti-citrullinated tenascin-c (c-TNC5) autoantibodies and antibodies against periodontal pathogen *Prevotella Intermedia* (27). Recent studies showed that antibody response to *Prevotella Copri* (component of gastrointestinal microbiota) seems to correlate with immune-response to GNAS and FLNA, two novel HLA-DR presented peptides auto-antigen identified directly in the synovial tissue and PBMCs of RA patients (28). An over-expansion of *P. Copri* in stool of new onset and chronic RA patients was identified, in both group the antibody response was specific for RA (29). With regard to other microorganisms, recent studies demonstrated a higher prevalence of anti-*Toxoplasma Gondii* IgG antibodies among RA patients (30), and an inverse association between high anti-*Epstein Barr virus* (EBV) and anti-*Parvovirus B19* IgG levels and the risk of developing ACPA positive RA (31).

Innate immune responses

In RA the dysregulation of immune system culminates in chronic inflammation, leading to progressive joint destruction. Growing interest has recently been given to professional phagocytes and DCs, as emerging critical cell populations in the pathogenesis of RA. In the peripheral blood of RA patients increased numbers of circulating monocytes have been reported. These cells are able to infiltrate the joints where they differentiate into synovial macrophages, that are highly activated in RA patients. Although different studies provide strong evidences for the implication of macrophages and DCs in the pathogenesis of RA, relatively little is known about the mechanisms behind their involvement. The use of experimental arthritis mouse models can be useful to clarify the role of professional phagocytes, DCs and other inflammatory cells in the pathogenesis of RA, providing a source for macroscopic and histological data simultaneously.

Using these *in-vivo* models, the disease can be macroscopically monitored over time via scoring systems. For histological examination, knee sections can be used for haematoxylin and eosin staining to visualise cellular infiltration as well as for tartrate-resistant acid phosphatase (TRAP) staining to identify osteoclast-like cells. Flow cytometric analysis allow to identify different population of myeloid cells infiltrating the synovium such as tissue resident macrophages, DCs and neutrophils. Different arthritis models such as CIA, antigen induced arthritis, and Streptococcal cell wall induced arthritis have been exploited to investigate different aspects of the pathogenesis of arthritis (32). In this regard, Dominguez *et al*. used K/BxN serum transfer-induced arthritis model, in order to investigate the impact of caspase-8 in development and progression of arthritis (33). In this model, the arthritis was induced by injection of arthritogenic serum from KRN and NOD mice (K/BxN mice), with the development of an inflammatory state characterised by severe, spontaneous, symmetric, erosive and chronic arthritis, that resembles the effector stage of RA. Moreover, K/BxN serum transfer-induced arthritis model is mediated by innate immune cells and relatively T and B cells-independent. Caspase-8 is a cysteine-aspartic acid protease and acts as initiator of apoptosis and suppressor of necroptosis. Previous studies evidenced that caspase-8 also maintains death-independent inflammatory processes, through a signalling axis that involves the suppression of receptor-interacting serine-threonine kinases (RIPKs). In addition, they observed opposing roles for caspase-8 signalling in lysozyme M-expressing cells and CD11 expressing cells in the joint, utilising two caspase-8 deletion constructs. Namely, caspase-8 in lysozyme M-expressing cells exacerbated the arthritis severity and hindered the resolution stage of arthritis itself. By contrast, caspase 8 in CD11-expression cells reduced the severity of effector phase of disease and delayed arthritis initiation. According to these results, caspase-8 is also implicated in the maintenance of synovial tissue-resident macrophages that can limit arthritis, and potentially controls the endocytic capacity of macrophages through the enhancement of CD206 expression. This receptor makes macrophages capable of endocytosing cellular debris arising from inflamed and damaged tissue, driving to the control of inflammation itself. In both caspase-8 deletion constructs, global deletion of RIPK3 abrogates the response to K/BxN serum transfer-induced arthritis, potentially trough other mechanisms independently of controlling cell death (33).

Additionally to their central function in the pathophysiology of synovial inflammation, monocytes/macrophages are at the origin of pathological bone erosion in RA. In fact, these cells contribute to both inflammation and cartilage and bone destruction through the production of degradative enzymes, cytokines and chemokines. In recent years, particular interest has been given to the investigation of cytokine production in monocytes/macrophages, in order to identify novel possible therapeutic targets. Recently, lactoferrin-containing immunocomplexes seems to be responsible of pro-inflammatory cytokines production by these cells, thereby contributing to the pathogenesis of autoimmune diseases such as RA (34). Lactoferrin (LTF) is a multifunctional iron-binding glycoprotein of the transferrin family and represents an important first line defense molecule against infection. This protein in known to be a target for humoral autoimmune reactions in humans, with the generation of anti-LTF specific autoantibodies (LTF-Abs) and LTF-containing immune complexes (ICs) (LTF-ICs). LTF-Abs are found in sera of patients with RA and other autoimmune diseases such as systemic lupus erythematosus (SLE), ulcerative colitis, Crohn's disease and ANCA-related vasculitis. Hu *et al*. reported the effectiveness of LTF-ICs to induce TNF-α and IL-1β production by human monocytes *in vitro*. On the contrary, control ICs or LTF and LTF-Abs alone were not able to elicit proinflammatory cytokine pro-

duction by monocytes. These results demonstrated that IC formation between human LTF and specific IgG in RA patients is essential to induce monocytes activation. Furthermore, the authors found that LTF-ICs utilised both CD32a (FcγRIIa) and membrane-anchored mCD14 (glycophosphotidyl inositol-anchored CD14 or GPI-anchored mCD14) to trigger monocyte activation in an internalization-, Toll-like receptor (TLR)-4- and TLR-9-dependent manner. The GPI-anchored mCD14 is a surface marker for phagocytic cells that relies on TLR-4 for signalling following sequester bacterial lipopolysaccharides (LPS) ligation. This element underlines the key role of the CD14/TLR4 complex (major components of the LPS signalling machinery) in the signalling during monocytes / macrophages activation. These results support the hypothesis that LTF-ICs may perpetuate local inflammation and contribute to the pathogenesis of autoimmune diseases by triggering activation of infiltrating monocytes or tissue macrophages *in vivo* (34).

While synovial macrophages are crucial in RA pathogenesis, the pathological role of of DCs is still unclear. Evidences from synovial tissues in RA indicates that DCs contribute to increase local infiltration of leukocytes and help initiation of disease by producing cytokines and presenting autoantigens to autoreactive T cells. Several types of DCs act as regulators of immune responses, so they are reported as tolerogenic DCs (TolDCs). TolDCs suppress autoreactive T cells in the thymus during central tolerance, limit effector T cells and promote regulatory T cell differentiation in the periphery.

Interestingly, Sendo *et al*. identified a new cellular population, $CD11b^{+}Gr1dim$ tolDC-LCs, in the severely inflamed lungs of SKG mice, an animal model of RA associated to an ILD, induced through injection of zymosan A (ZyA). The authors demonstrated that GM-CSF, produced by both helper T (Th) cells and innate lymphoid cells (ILCs), stimulates the differentiation of Myeloid-derived suppressor cells (MDSCs) into $CD11b^{+}Gr1dim$ tolDC-LCs, that serve as suppressor of ILD in SKG mice. The molecule CD11c is constitutively expressed on the cellular surface of DCs, that are potent antigen-presenting cells with an essential role in initiating adaptive immune responses.

$CD11b^{+}Gr1dim$ tolDC-LCs can be classified as a subset of tolDCs, phenotypically different from tipical tolDCs, expressing high levels of PD-L1 and low levels of CD80. This new subset of tolDCs express high levels of both PD-L1 and CD80. The molecular mechanisms of ILD suppression by $CD11b^{+}Gr1$ dim to lDC-LCs are still to be elucidated (35).

Another important feature of innate immune cells is the activation of antimicrobial pro-inflammatory immune responses through TLRs, which can initiate different signalling pathways. These signalling pathways are also implicated in the metabolic switch from mitochondrial respiration to anaerobic glycolysis, that occurs under hypoxic conditions, even during inflammatory states. For example, TLR-4, TLR-2 and TLR-9 signalling induces an increase in glycolytic rate and glucose consumption in DCs, while activation of TLR-4, TLR-2 and TLR-6 in macrophages promotes an M1 phenotype, resulting in increase in mitochondrial reactive oxygen species (ROS) and a dependency on glycolysis.

Previous studies have shown increased mitochondrial DNA mutation frequency and mitochondrial dysfunction in the RA joints. These effects are associated with oxidative stress state, angiogenesis processes, pro-inflammatory cytokines production and activation of the NLRP3 inflammasome. In this regard, McGarry *et al*. demonstrated alterations in mitochondrial function in the RA joint in response to TLR-2 signalling, in parallel with a dysregulation of glucose metabolism (36). The authors investigated the effect of TLR-2 activation on mitochondrial function and bioenergetics in primary RA-FLS, identifying a link between TLR signalling, ROS and mitochondrial dysfunction in these cells. The authors demonstrated that TLR-2 induced random mitochondrial point mutations in RA synovial tissue and primary RA-FLS, resulting into mitochondrial mutator phenotype which may be involved in the regulation of inflammatory responses. In parallel, TLR-2 activation induced mitochondrial dysfunction in RA-SF *in-vitro* system, such as reduction of mitochondrial membrane potential and increase of ROS and 4-hydroxynonenal (4-HNE), that is a marker of lipid peroxidation. In previous studies ROS had been reported as primary source of mitochondrial mutagenesis and dysfunction, since mitochondrial genome is highly susceptible to oxidative damage. Meanwhile, the increase in 4HNE, in parallel to a decrease in mitochondrial membrane potential, is consistent with previous studies showing that lipid peroxidation induces the mitochondrial membrane to become more permeable to protons, dissipating the mitochondrial membrane potential. This is a consequence of the reaction of ROS with lipids. Taken together, these data suggest that TLR-2-induced ROS production can drive mitochondrial mutation, lipid peroxidation, and damaging of the mitochondrial membrane. The damaging of mitochondrial membrane can further induce ROS production, resulting in a vicious cycle of mitochondrial dysfunction, which can drive inflammation (36).

Among all cells implicated in the pathogenesis of RA, neutrophils exert the greatest cytotoxic effects, due to their ability in releasing degradative enzymes and reactive ROS. Neutrophils also contribute to the production of cytokine and chemokine cascades associated to inflammatory processes and regulating immune responses via cell-cell interactions.

A particular subpopulation of peripheral blood mononuclear cells (PBMC), known as low-density granulocytes (LDGs), was firstly identified in the blood of SLE patients in 1986 and subsequently described as a group of cells expressing surface markers specific for mature neutrophils ($CD15^{high}/CD14^{low}/CD10^{+}/CD16^{+}$) with a gene expressing profile characteristic of immature neutrophils. During early stage of neutrophils differentiation, granule protein genes and cell-cycle checkpoint genes are expressed at higher levels, while expression of genes codifying apoptotic

molecules, cytokines, chemokines and their receptors are down-regulated.

Wright *et al*. investigated the characteristics of a subpopulation of LDGs from RA patients, in order to determine if they are functionally different from RA neutrophils. Interestingly, these RA LDGs expressed elevated level of transcripts for granule proteins, including elastase and myeloperoxidase (MPO), and also expressed cell-cycle genes, including cyclin-dependent kinase (CDK2, CDK4, CDK6), resembling an immature phenotype of neutrophils. In parallel, apoptosis-regulating genes were expressed at lower levels in LDGs, translated into a significantly lower rate of apoptosis in this cellular population with a decreased response to TNF-α *in-vitro* system. In fact, the expression of cytokines and cytokines receptors, especially TNF receptors (TNFRs) were lower in RA LDGs compared with RA neutrophils; providing an explanation for the lack of response to TNF-inhibitors therapy in some RA patients. Further studies are required to clarify the contribution of these cells in RA pathogenesis and to understand whether LDGs represent mature neutrophils or a different phenotype of neutrophils (37). Additionally to the well-recognised cytotoxic and immunoregulatory functions in RA, neutrophils may also provide a source of the autoantigens, contributing to the genesis of autoimmune processes in this disease. In fact, emerging evidence suggests that RA neutrophils can release neutrophil extracellular traps (NETs) containing chromatin associated with granule enzymes, which not only kill extracellular microorganisms but also provide a source of autoantigens. NETs formation has been identified as a bridge between innate and adaptive immune responses in autoimmunity. An array of cytoplasmic and extracellular citrullinated proteins has been described among NET components. They can act as neoepitopes in loss of immune tolerance. These citrullinated proteins are generated by PADs, which replace arginine with citrulline residues, within neutrophils. Although PADs are reported to catalyze citrullination in inflammatory conditions, the precise role of PADs in RA is unclear. Within this group of proteins, PAD4 contributes to inflammation in different murine models of RA; it is also associated to LPS-induced histone citrullination and NET formation. Meanwhile, PAD2 is important for citrullination in healthy tissues, is present in NETs and correlates with disease activity in RA.

Bawadekar *et al*. used an experimental animal model of RA (TNF-α-induced inflammatory arthritis) to identify the roles of PAD2 and PAD4 in citrullination and NET formation in inflamed joints.

In mice with TNF-α-induced arthritis, there was an increased citrullination in inflamed joints, which persisted in absence of PAD4, but not in absence of PAD2 that was not required for NET formation. According to these results It seems that PAD4 is not crucial for generation of citrullinated proteins in TNFa-induced arthritis, although It may contribute to RA in other ways. On the contrary, PAD2 appeared to be required for joint citrullination in TNF-α-induced arthritis, without a major role in NETs formation, suggesting the presence of other possible main sources of citrullinated antigens, such as immune-mediated membranolysis-induced hypercitrullination, potentially catalyzed by PAD2 (38). Nonetheless, the importance of NETosis is underlined by spread evidences, since this phenomenon correlates with presence and levels of ACPA and with systemic inflammation. Furthermore, NETosis is enhanced in the peripheral blood and the synovium of patients with RA.

The generation of ACPAs in early phases of RA has been recently investigated in a study performed by Demoruelle *et al*. Emerging data suggests that RA related autoimmunity is initiated at a mucosal site and that ACPAs may be initially generated at a mucosal surface. In order to clarify the role of lung mucosa in the early stages of RA related autoimmunity and explore ACPAs generation in the lung, Demoruelle *et al*. investigated samples of induced sputum and serum obtained from RA patients and RA-free first-degree relatives (FDRs) (39). Their study provided evidence that ACPA isotypes as measured by anti-CCP are present in the sputum of a portion of FDRs and subjects with early disease, suggesting that the lung may be a site of anti-CCP generation in this population.

Increased levels of IgA and IgG anti-CCP were detected in RA patients (70%) and in FDRs (25%), including a portion of FDRs who were serum anti-CCP negative. In the FDRs, elevations of sputum IgA and IgG anti-CCP were associated with elevated cell counts and NET levels in the sputum. These findings show that local airway inflammation and NET formation are associated with increased ACPAs in the lung, suggesting that NETosis may drive ACPA generation in the respiratory tract of FDRs who are at an elevated risk of developing RA.

Moreover, ACPA isotype positivity was present in a proportion of FDRs in the absence of serum ACPA positivity, even if the number of FDRs demonstrating sputum ACPA positivity exceeds the number that statistically will develop classifiable RA. This suggests that local ACPA formation may be necessary but not sufficient to develop a systemic state of the disease (39).

Taken together, these results support the hypothesis that lung could play an important role in the early stages of RA development.

In addition, they also characterised in the sputum of subjects at risk for RA (at-Risk) the reactivity of antibodies to individual citrullinated and non-citrullinated proteins/peptides as well as associations with NETosis. The authors, evaluating the individual antibody responses in RA subjects and in subjects at-Risk for the future development of RA, based on familial or serologic risk factors, concluded that sputum antibody reactivity to particular citrullinated and non-citrullinated proteins/peptides is specific for at-risk and RA subjects. In addition, the levels of sputum antibodies to citrullinated antigens was significantly higher in at-Risk and RA subjects compared to controls. Within the at-risk subjects, the most prevalent sputum antibody responses to citrullinated proteins/peptides were directed to cit-fibrinogen, cit-apolipoprotein E and cit-fibronectin, even in serum ACPA negative at-Risk subjects,

suggesting that these proteins may represent the earliest antigen targets of antibodies generated in the lung. On the other hand, within RA subjects, the most prevalent sputum antibodies to citrullinated proteins/peptides were directed to cit-filaggrin, cit-histone 2A, cit-histone 2B, cit-fibrinogen, cit-fibronectin and cit-clusterin.

In the serum of preclinical and early RA subjects, antibodies to non-citrullinated/native proteins have been detected in addition to anti-citrullinated antibodies, suggesting that autoimmunity may be initially directed to native proteins, subsequently to citrullinated epitopes through epitope spreading. Demoruelle *et al*. sought to explore antibody reactivity to both citrullinated and non-citrullinated antigens during different phases of RA development. They identified antibodies with both a citrullinated and non-citrullinated antigen counterpart, but certain antigens appeared to induce a more citrullinated specific reactivity. Particularly, the most citrullinated specific sputum antibodies in at-risk subjects were directed to fibrinogen, vimentin and the peptides fibrinogen A and apolipoprotein A1. Meanwhile, a high level of cit-specificity in RA patients was demonstrated for the proteins fibrinogen, histone 2A, histone 2B and vimentin and the peptides histone 2A/a-2 and fibrinogen A. A correlation between sputum levels of NET complexes and several sputum antibodies to citrullinated and non-citrullinated proteins/peptides in at-Risk subjects has been reported. The sputum NET levels were significantly associated with antibodies to cit-fibrinogen, cit-apolipoprotein A1 and fibrinogen A, which had also demonstrated high sputum citrullinated-specificity in at-Risk subjects. In the case of antibodies to histone and vimentin peptides, sputum NET levels significantly correlated only with the citrullinated counterparts. Interestingly these two citrullinated-proteins have been identified in the protein cargo of NETs induced in RA patients' neutrophils (39). Similarly to the initiation of inflammation, there is a growing appreciation that the resolution of inflammation is an intricate and active process.

It is well known that RA is characterised by a chronic inflammation, as a result of the inability to resolve an immune response. The physiological pathways involved in the resolution of arthritis are still incompletely understood, but the detection of immune components that control the resolution process may be key to novel therapies. In recent years, there has been growing interest in the biology of newly discovered immune cells, ILCs, since they are considered crucial mediators of tissue remodeling and repair. ILCs are the counterpart of Th lymphocytes and can orchestrate inflammation, innate and adaptive responses. For example, they can closely interact with stromal cells, leading to their up-regulation of adhesion molecules and production of chemokines. These cells are also capable of secreting different mediators in adult lymphoid tissues. In particular, ILC2, the counterpart of Th2 cells, have been reported to be crucial in tissue repair through the function of cytokines and soluble mediators. Rauber *et al*. found that ILC2 induce the resolution of inflammation in RA via the production of IL-9, which has been identified as a master molecule in regulating resolution of arthritis and preventing the chronicisation of arthritis itself (40).

The authors investigated the function of IL-9 in the context of antigen-induced arthritis, finding that ILC2 are the major source of IL-9 during the resolution phase of arthritis and play a pivotal role in the promotion of regulatory T cells (Treg) with the suppressive activity. In fact, stimulation of ILC2 with IL-9 induced an up-regulation of the Treg-receptor-associated ligands GITRL and ICOSL, which are known to increase the suppressive capacity of Treg. This observation is in line with previous reports that ILC2 produced IL-9 acts in an autocrine loop to promote ILC proliferation. The described cellular pathway effectively reduced tissue damage, with preservation of cartilage integrity, and decreased bone erosions, translated into accelerated resolution of arthritis. Supporting these evidences, high numbers of ILC2 expressing IL-9 are detectable in the joints and in the circulation of RA patients in remission phase of the disease. By contrast, the absence of IL-9, in genetically deficient mice, impaired ILC2 proliferation and Treg, leading to persistent synovitis and excessive degradation of cartilage and bone (40).

The adaptive immune system

The adaptive immune system is a leading actor in the development of RA and the imbalance of effector (eff) and regulatory (reg) lymphocytes is a hallmark of disease pathogenesis. The disruptive effects of T helper (h) 17 cells in RA have been further supported over the last year by studies demonstrating the association of IL-17 and intra-articular IgA secretion (41), the capability of Th17 cells to trigger specific B lymphocyte clones to produce autoantibodies in the preclinical RA phase (42), the key role of IL-21 in orchestrating bone damage together with TNF-α (43) and the IL-17-induced mitochondrial dysfunction in FLS (44). Data from animal models unmasked novel mechanisms leading to a modulation of T cell subsets such as the anti-inflammatory effects of IL-38 on Th17 cells (45) or the lack of involvement of CTLA-4 in immune priming in CIA (46). In the field of Th17 cells, interesting data from Lin *et al*. demonstrate that YY1, a "Yin Yang" transcription factor involved in cancer development and progression, is able to enhance IL-6 production and in consequence Th17 cell commitment in CIA mice (47). Conversely, over-expression/stimulation of programmed cell death 5 (PDCD5), sialic acid-binding Ig-like lectin-9 (Siglec-9) or leukocyte-associated Ig-like receptor-1 (LAIR-1) restores the Treg/Teff ratio by the increase of Treg cells and the reduction of Th1 and Th17 cells in the same experimental RA model (48–50).

Semaphorin 7A is a powerful inducer of Th1 and Th17 cells (51), while Galphaq-containing G protein, a member of Gq/11 class, is a strong enhancer of Th1 cells in experimental model (52). Oncostatin, a cytokine belonging to the IL-6 family which action is still unclear, demonstrated to be anti-inflammatory in CIA by hampering Th17 cell commitment through the modulation of SOCS3, STAT3, and STAT5 (53). As

far as B lymphocytes are concerned, new insights into the mechanism of tolerance breaking and autoantibody production have been provided by Dekker *et al*. who investigated the response of autoreactive B cells against proteins that underwent post-traslational modifications and observed that not only self, but also foreign anti-carbamylated proteins can trigger an aberrant autoimmune response against self carbamylated proteins (54).

Over the last year, several papers provided evidences about novel modulators of the T lymphocyte subsets also in the human counterpart. Cell-to-cell contact is a key event allowing crosstalk and cognate interaction of immune cells. Yang *et al*. demonstrated that Th17 cell differentiation requires cell-to-cell contact via CD147 with activated monocytes (55), while Mori *et al*. demonstrated that this differentiation, as well as the commitment into Th1 cells, can also be induced by cell-to-cell contact of FLS and naïve T lymphocytes via adhesion molecules (56). Interestingly, while in normal conditions FLS would inhibit Th1 cell proliferation via the indoleamine 2,3, dyoxigenase pathway and prevent their abnormal proliferation, in RA Th17 cells can interfere with such pathway thereby protecting Th1 cells from this inhibitory mechanism (57). DCs are another important cell type involved in this process and integrin αv a pivotal mediator. In fact, integrin αv is up-regulated on the surface of RA DCs and drives the differentiation of Th17 cells through the induction of TGF-β (58).

The PD-1 receptor is a crucial modulator of T cell activation, and Treg cells are able to release its soluble isoform (sPD-1). Two studies reported an increase of sPD-1 in RA. However, while Ren *et al*. provided evidences of a possible suppressive effect of this molecule on Teff (59), Bommarito *et al*. demonstrated a certain degree of resistance of RA Teff cells to sPD-1 dependent suppression (60). In addition, Rao *et al*. identified a novel T cell population in RA synovial tissue that besides high surface levels of PD-1 also displays factors mediating B-cell help and several chemokine receptor, but lacks CXCR5, the main marker of follicular helper T cells. These peculiar T lymphocytes have been defined peripheral helper T cells (61).

The effects of prostaglandin E2 (PGE2) on Treg cells have been described for the first time. PGE2 is able to profoundly affect Treg cells in many ways including the down-regulation of FoxP3, CTLA-4 and glucocorticoid-induced tumour necrosis factor receptor-related protein (GITR) and the inhibition of IL-10 release (62).

Another intriguing aspect that has been investigated is T cell recruitment at RA target sites. Wen-Xiu *et al*. identified a peculiar T cell subset, Vδ2 T cells, that express chemokine receptors CCR5 and CXCR3, produce pro-inflammatory cytokines and accumulate in RA synovial membrane upon TNF stimulation. Of interest, treatment with TNF inhibitors strongly down-regulated the expression of these chemokine receptors hence interfering with the migration of Vδ2 cells (63). Another study, provided the first evidence of an *in-vitro* chemotactic activity of the insulin growth factor binding protein 6 (IGFBP6) on RA T cells that could be partially inhibited by dexamethasone. As additional findings, higher expression of IGFBP6 in RA compared to osteoarthritis (OA) synovial tissue as well as in RA compared to normal subjects peripheral blood were observed (64). Finally, thymus and activation-regulated chemokine (TARC), a chemotactic factor exerting its effect on CCR4-expressing cells, was found to be increased in RA SF with DCs being the major source. Given that T lymphocytes express CCR4, DCs also display chemotactic activity on T cells via TARC (65).

Moving to B lymphocytes, in recent years an increasing number of article supported the hypothesis that besides their capability to produce antibodies, B cells can also act as antigen presenting cells (APC). In this regard Shimabukuro-Vornhagen *et al*. identified a B cell subset with a peculiar range of surface markers able to strongly stimulate T lymphocytes and that is expanded in RA patients (66). Very interesting data come from the study of Hu *et al*. with regard to aberrant function of Breg cells in RA. The authors demonstrated that B10 cells, that in normal conditions counteract chronic inflammation, abnormally produce RANKL in RA and therefore are involved in the development of bone erosion. Interestingly, such phenotype is closely correlated with the severity of the disease, being less prevalent in patients undergoing remission (67).

Conclusion

During the last year, relevant findings in the field of RA pathogenesis have been described. In particular, new insights come from studies on the innate and adaptive immune system, including cells, soluble mediators, adhesion molecules and intracellular pathways. Phenotypes and functions of T and B lymphocytes have been better characterised and novel roles of the newly discovered innate lymphoid cells (ILC) has been proposed. Future studies will be needed to better understand the mechanisms underlying RA, in order to develop novel and more specific disease-modifying therapies.

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