One year in review 2017: pathogenesis of rheumatoid arthritis

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ABSTRACT
Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease influenced by both genetic and environmental factors. It has been postulated that a high-risk genetic background, in combination with epigenetic marks and environmental exposures, leads to a cascade of events inducing synovitis and consequent destructive arthritis. The clinical picture of joint involvement in RA is the result of chronic inflammation of the synovium, characterised by interactions of resident cells such as fibroblast-like synoviocytes (FLS) with cells of the innate (e.g. macrophages, dendritic cells, mast cells and NK cells, neutrophils) and adaptive immune system (e.g. B and T lymphocytes). Soluble mediators, autoantibodies, adhesion molecules and signal transduction pathways are all involved at different stages of the disease. Here we report a Medline search of articles in English published in the PubMed database from 1st January 2016 to 31st December 2016 giving new insights in the field of RA pathogenesis. All articles were critically analysed in order to provide an improved understanding of the mechanisms underlying RA and to contribute to identify new targets for the development of novel disease-modifying therapies.

Genetic aspects
Recent advances in genome-wide association studies and subsequent meta-analyses have led to the identification of many alleles that govern RA susceptibility (3). Although predominant associations with the HLA-DRB1 locus were identified decades ago, recent data have revealed additional insights into the likely causative variants within HLA-DRB1 as well as within other HLA loci that contribute to disease risk. Data obtained from genome-wide association analysis are variable according to the population evaluated. In the southern Indian population, by studying the presence of the shared epitope in the HLA-DRB1 alleles, Konda Mohan et al. showed that HLA-DRB1*01, *04, *10 and *14 alleles are related to RA, while HLA-DRB1*03, *07, *11 and *13 protect against the development of the disease (4). In a Swedish population, Jang et al. evaluated whether known RA susceptibility genes, HLA-DRB1 amino acids, and haplotypes either individually or integrated into a risk score, pre-

Competing interests: none declared.
dicted treatment response to inhibitors of tumour necrosis factor (TNF	xapy. Although all of these were strong predictors of RA, they found that a high
genetic risk score was not associated with good response in either overall RA
or stratified by anti-citrullinated protein antibody (ACPA) status. According to
the results, the authors concluded that there is no strong evidence supporting a
significant role of RA risk genes in the response to TNFi treatment. In addition,
one of the single nucleotide polymorphisms (SNPs), amino acids, or haplo-
types examined in their study seem to be meaningful independent predictors
of response to TNFi therapy, although weak associations cannot be ruled out
(5). Particular attention has been paid to the association between ACPA and
HLA-DR alleles. These autoantibodies are highly prevalent in RA patients
and are strongly associated to a specific set of HLA-DR alleles, including HLA-
DRB1*04:01, *04:04, and *01:01. Gerstner et al. demonstrated that the levels of
ACPAs towards the dominant citrullinated B cell epitope of α-enolase
are significantly elevated in HLA-
DRB1*04:01-positive RA patients.
Furthermore, they identified α-enolase-
derived T cell epitopes and demonstrated
that native and citrullinated versions of several peptides bind with different
affinities to HLA-DRB1*04:01, *04:04, and *01:01 (6). In addition to the HLA-
DRB1 alleles, genomic stratification by expression of HLA-DRB4 alleles led to
identify differential innate and adaptive immune transcriptional patterns, which
might predict the response to therapy, namely methotrexate (MTX) in early
RA (7). Some interesting results on the association between polymorphisms of
HLA loci with RA onset or response to therapies also come from studies performed
in animal models of RA. In collagen-induced arthritis (CIA) the HLA-G2 protein, involved in maternal-
fetal immune tolerance, has been found to be a useful target for the treatment
of RA, specifically binding paired Ig-
like receptor (PIR)-B (PIR-B), only expressed by antigen presenting cells
(APC). In their study, Takahashi et al. were able to demonstrate the immu-
nosuppressive effect of the domain-
deleted dimer of HLA-G2 isoform in the
CIA model, thereby postulating that
this effect might be due to the suppression
of antigen presentation by HLA-
G2 proteins (8). In addition to the HLA loci, more than 100 common variants in non-HLA loci have been implicated in RA susceptibility. For example, polymorphisms in signalling transducers and activators of transcription- (STAT)-4 and inter-
leukin- (IL)-10 genes confer susceptibility
to RA and SNPs in PSORS1C1, PTPN2 and MIR146A genes were asso-
ciated to various extents with a severe
disease phenotype in terms of autoantibody status and radiographic damage (9). In parallel, Lee et al. (10) investigated the associations between
K469E and G241R polymorphisms of the intercellular adhesion molecule-1
(ICAM-1) and vasculitis or RA, demon-
strating that the G241R polymorphism
is associated with susceptibility to RA as well as Behçet’s disease. The association of the non-coding triallelic dinucleotide polymorphism CCR6DNP
with risk for RA has also been hypothesised. This allelic variant seems to correlate with the expression of the C-C chemokine receptor-6 (CCR6). By us-
ing transcription activator-like effector
nulease (TALEN) gene editing, Li et al. (11) confirmed that CCR6DNP regulates CCR6 and that is a causal variant through which the Poly(ADP-ribose)
polymerase-1 (PARP-1), multifunc-
tional nuclear protein involved in a va-
riety of cellular functions, regulates this
receptor.
Several candidate gene variants have been identified by genetic mapping in
RA. However, RA as well as other au-
toimmune diseases cannot be explained
by genetic susceptibility alone. The term ‘epigenetic’ refers to inheritable and potentially reversible changes in DNA and chromatin that regulate gene expression without altering the DNA sequence. Among the mechanisms in-
volved in epigenetic regulation, DNA
methylation, histone modification, and non-coding RNA-mediated regulation
are currently under intense investiga-
tion. Therefore, a novel approach is to
study the high-risk genetic background
in combination with epigenetic modi-
fications. This combined approach al-
lows to better understand the mecha-
nisms underlying the innate and adap-
tive immune responses during RA.
Gene-analysis combined with an epige-
netic regulation study has been recently investigated in the regulation of FLS.
These are synovial intimal lining cells
which display unique aggressive behav-
iour, invading the articular cartilage
and promoting inflammation through the
production of cytokines, chemokines,
and proteases. The PTPNI1 gene, en-
coding the tyrosine phosphatase SHP-2,
is overexpressed in RA FLS compared
to those from osteoarthritis (OA) pa-
tients and promotes RA FLS invasive-
ness. Using computational methods,
Maeshima et al. identified a putative enhancer in PTPNI1 intron 1, which contained a glucocorticoid receptor-
bounding (GR-binding) motif. This re-
gion displayed enhancer function in
RA FLS and contained two hypermeth-
ylation sites in RA compared with OA
FLS. The authors finally demonstrated how abnormal epigenetic regulation of a pathogenic gene determines RA FLS
behaviour and targeting SHP-2 or the
SHP-2 pathway could be a therapeutic
strategy for RA (12). This approach
may be used to dissect the critical ab-
normals of RA FLS and define can-
didate molecular or pathway targets for
therapeutic intervention.

Environmental factors
The development of RA cannot be fully explained by genetic factors and epige-
netic mechanisms. It has been proposed that many environmental components,
including smoking, hormones, infec-
tion and microbiota are critically in-
volved in the induction of the disease in genetically predisposed individuals.

Cigarette smoke
Gene-environment interactions are known to play a key role in the develop-
ment of RA. Exposure to cigarette smoke (CS) is probably one of the
strongest environmental risk factors
associated with the disease. CS might
exert a range of complex modulatory
effects on both innate and adaptive
immune systems. In recent years, several
studies have tried to clarify the mecha-
The immune system and its contribution to the development of the disease (13). Interestingly, Sparks et al. performed a study to prospectively evaluate genetic, environmental, and serologic risk factors in a cohort of first-degree relatives (FDRs) of RA patients. This group of subjects is at increased risk of RA based on positive family history, but many of them were asymptomatic and seronegative, so are likely further away from developing RA. In this study, CS resulted to be an important, and potentially modifiable risk factor during preclinical transitional phases of RA. In fact, FDRs smoking more than 10 pack/years and younger than 50 years of age represent the group with the highest risk of developing inflammatory joint signs (14). Additional information on gene-smoke interaction in RA came from Immunochip (ICHIP), a custom-made Illumina Infinium array, including 195586 genetic markers from 5043 samples. In this study multiple gene-environment interactions have been identified in autoantibody-positive RA, taking into account all genetic variations in the ICHIP scan and using CS as environmental exposure. Extended gene variation patterns involved in gene-smoking interaction in ACPA-positive, but not ACPA-negative RA have been identified. Notably, variants in HLA-DRB1 and those in additional genes within the MHC class II region, but not in any other gene regions, showed interaction with CS. Taken together, these results might be useful to design further functional studies on the potential effects of MHC class II-dependent immune activation in ACPA-positive RA following CS exposure (15). The effects of CS on the immune response have been widely studied in animal models. By using DEF6 deficiency mice, Weng et al. investigated the effects of CS on the function of Th helper (Th)17 cells, one of the key effector subsets implicated in RA pathogenesis. They have demonstrated that the direct exposure of CD4+ T cells to CS extract enhances the production of IL22, a member of the IL10 cytokine family produced by several different cellular sources including Th17 cells, Th22 cells, NK cells, lymphoid tissue inducer (LTI) cells and γδT cells. After treatment with CS extracts, they also observed decreased ROCK2 activation and phosphorylation in T cells of interferon regulatory factor-4 (IRF4), a known negative regulator of IL22. Using a GEF pull-down assay they further demonstrated that this effect was associated with a reduction in the activation of ARHGEF1, an upstream regulator of the RhoA-ROCK axis (16), confirming the complex interaction between gene and environmental factors in RA and the regulatory role of CS in the development of the disease.

Microbiota
Growing evidence has shown that the changes in the composition and function of mucosal microbiota are closely related to RA. Several studies have demonstrated that the composition of gut microbiota is altered (dysbiosis) in RA patients. Through the analysis of faecal samples, Chen et al. showed that patients with RA exhibited decreased gut microbial diversity compared with healthy individuals, which correlated with disease duration and autoantibody levels. A taxon-level analysis suggested an expansion of rare taxa, namely Actinobacteria, in patients with RA. By using predictive models based on the random forests algorithm, they also demonstrated that the three genera, Collinsella, Eggerthella, and Faecalibacterium segregate with RA and that the abundance of Collinsella strongly correlates with high levels of alpha-aminoadipic acid and asparagine as well as with the production of the pro-inflammatory cytokine IL17A. In addition, Collinsella seems to modulate gut function by altering its permeability and enhancing the severity of the disease (17). Besides differences in the composition of the microbiota, the current research is focusing on characterisation of the functional role of microbial components in RA. For this purpose, Maeda et al. analysed the composition of faecal microbiota of patients with early RA, using 16S ribosomal RNA-based deep sequencing. Following inoculation of faecal samples obtained from RA patients into germ-free arthritis-prone SKG mice, which spontaneously develops Th17-mediated autoimmune arthritis, they studied the immune responses in this animal model. They also analysed whether the lymphocytes of SKG mice harbouring microbiota from RA patients react with the arthritis-related autoantigen 60S ribosomal protein L23a (RPL23A). These in-vivo and ex-vivo approaches allowed to demonstrate that dysbiosis increases sensitivity to arthritis via activation of Th17 cells in the intestine, and that the autoreactive SKG mouse T cells are activated by dysbiotic microbiota, leading to the development of joint inflammation. Therefore, the authors concluded that Prevotellaceae-dominant intestinal microbiota seems to be an environmental factor that promotes genetically susceptible T cells to induce arthritis, supporting the direct contribution of dysbiosis in triggering arthritis development in genetically susceptible mice (18). Besides Th17 cells, other T-cell subsets seem to be involved in the regulation of gut microbiota in RA. By using the K/BxN autoimmune arthritis model, Block et al. have shown that the gut microbiota regulates arthritis through a sub-set of Th cells, named follicular helper T cells (Tfh), and that this regulation is independent of the Th17 subsets. They have hypothesised that Tfh cells promote activation and differentiation of B cells through the production of inflammatory cytokines such as IL21 and IL4 (19). In parallel, the immune relevance of another microbial component, Prevotella copri, has been investigated. This is an intestinal microbe over-expanded in stool samples of patients with new-onset rheumatoid arthritis (NORA). In peripheral blood mononuclear cells (PBMC) purified from NORA patients, an HLA-DR-presented peptide from a 27-kD protein of P. copri (Pc-p27) has been identified. In 42% of these patients, the Pc-p27 seems to exert a regulatory role on the adaptive immune response by stimulating a Th1 phenotype. In both NORA and established RA groups, a subgroup of patients producing IgA antibodies directed to Pc-p27, which correlated with Th17...
cytokines and ACPA, has been identified. The rest of patients produced IgG antibodies directed to P. copri, which were associated with Prevotella DNA in synovial fluid (SF), P. copri-specific Th1 responses, and lower prevalence of ACPA. Interestingly, the production of antibodies to P. copri were rarely found in patients with other rheumatic diseases or in healthy population, suggesting that the immune response to P. copri is specific for RA (20).

The use of various animal models to highlight mechanisms underlying RA pathogenesis often provide conflicting results. Therefore, studies in human along with studies in animal models are required in order to better understand how environmental factors modulate immune responses in RA and contribute to the development of the disease.

**Innate immune response**

**NETosis and autophagy**

In recent years, particular interest has been given to the contribution of neutrophil extracellular trap (NET) formation (NETosis) to the breaking of immunological tolerance and the maintenance of autoimmunity and inflammation in RA. NETosis is an important first line defense mechanism of neutrophils, occurring at an early step of the inflammatory cascade. NETs trap and restrain invading pathogens and use their highly localised focus of antimicrobial granular peptides to degrade virulent factors and even kill microorganisms. This process consists of the release of intracellular components, including DNA, histones and an array of proteins that all together create a net-like structure outside the cell. Although NETosis is a pathogenic mechanism shared by several autoimmune diseases, NET components and their effects on the immune system might diverge in different conditions. In RA, neutrophils from PB and SF display an increased NETosis that can be appreciated in basal conditions or after stimulation with serum antibodies or inflammatory cytokines. In RA, neutrophils exhibit increased spontaneous NETosis, associated with elevated reactive oxygen species (ROS) production, enhanced neutrophil elastase and myeloperoxidase (MPO) expression, nuclear translocation of protein arginine deiminase-4 (PAD4), PAD4-mediated citrullination of H3, and altered nuclear morphology. Among NET components, a range of cytoplasmic and extracellular citrullinated antigens, well-established targets of ACPA, have been described. These components might be the target for autoantibodies and immune complex formation and might act as inducers of further NETosis in RA as well as juvenile idiopathic arthritis (21).

Corsiero et al. provided novel evidence that B cells differentiated within synovial ectopic lymphoid structures (ELS) in the RA joints frequently target deaminated proteins, which could be generated during NETosis of RA synovial neutrophils, including histones. They demonstrated that 40% of RA synovial B cells from ELS displayed reactivity against citrullinated histones, mainly H2A and H2B (citH2A/H2B). Therefore, anti-citH2A/H2B-reactive RA recombinant monoclonal antibodies-ramAbs, but not anti citH2A/H2B negative, selectively recognised NET from PB and/or RA joint neutrophils. Thus, NET could represent a source of citrullinated antigens, fueling the ACPA autoimmune response within the RA synovium (22).

In parallel to human studies, new insights into the pathogenic role of NET in RA come from research conducted in the CIA model. Papadaki et al. have demonstrated that NET are involved not only in the innate immunity, but also in the adaptive counterpart. In fact, they showed that NET are able to promote pathogenic IFN-γ-producing Th1 immune responses by modulating the activation and maturation of dendritic cells (DC). In particular, they provided evidence for a direct immunogenic ability of NET to increase the DC co-stimulatory molecules, CD80 and CD86 as well as the secretion of the pro-inflammatory cytokine IL6 (23). It is still unclear which mechanisms are involved in the NET recognition by DC, but a role of Toll-like receptor-9 (TLR9) in this process has been postulated. The inhibition of NETosis in the in-vivo model using Cl-amidine (a pan-PAD inhibitor), significantly diminished the severity of arthritis and delayed disease onset, strengthening the hypothesis of a strict connection between citrullination and NETosis in RA.

In parallel to NETosis, autophagy has been proposed as a cellular mechanism involved in the pathogenesis of RA (23). This is an endogenous process necessary for the turnover of organelles, the maintenance of cellular homeostasis and the regulation of cell fate. This process seems to contribute to the progression of various rheumatic diseases, including RA. During autophagy, parts of the cytoplasm and organelles are encapsulated in double-membraned vacuoles called autophagosomes, which finally fuse with lysosomes to degrade the incorporated material using acidic hydrolyases. By using in-vitro systems, Sorice et al. demonstrated a role of autophagy in the generation of citrullinated peptides. Following treatment of FLS with tunicamycin or rapamycin, both inducers of autophagy, the activation of PAD4 occurs, with consequent protein citrullination and formation of vimentin, α-enolase and filaggrin as the main citrullinated proteins (25). This cellular mechanism seems to be altered also in T cells. Van Loosdregt et al. investigated whether autophagy is dysregulated in CD4+ T cells of RA patients, resulting in disturbed T-cell homeostasis. They demonstrated that the rate of autophagy is significantly increased in CD4+ T cells from RA patients, and that increased autophagy is also a feature of in-vitro activated CD4+ T cells. The increased apoptosis resistance observed in CD4+ T cells from RA patients was significantly reversed upon autophagy inhibition (26). Therefore, according to these recent findings, understanding in detail the mechanisms of autophagy in RA and its role in the development of the disease is relevant for designing more accurate and specific therapeutic approaches.

**Innate immune cells**

A tight interaction between resident cells and innate immune cells takes place during chronic inflammation of RA synovium. This leads to structural changes of the synovial tissue and...
subsequent joint destruction. Besides neutrophils, myeloid cells seem to exert relevant roles in the induction and progression of inflammatory processes (27).

Stimulation of colony stimulating factor-1 receptor (CSF1R) by colony stimulating factor-1 (CSF1), also known as macrophage colony-stimulating factor (M-CSF), promotes survival, proliferation and differentiation of myeloid cells, including monocytes, macrophages and osteoclasts. CSF1 has an essential function in joint destruction, as it is required for osteoclastogenesis and TNF-induced osteolysis. Recently, IL34 was discovered as a novel ligand of CSF1R. CSF1 and IL34 share structural but not sequence homology, and have largely overlapping effects on CSF1R downstream signalling, regulation of monocyte survival, macrophage polarisation, and osteoclastogenesis. Emerging studies indicate that IL34 levels are increased both in serum and in SF of RA patients, and are strongly associated with rheumatoid factor (RF) and ACPA levels. The contribution of CSF1 and IL34 signalling to CSF1R in RA has been recently proposed. By blocking CSF1R using specific antibodies that prevent binding of both CSF1 and IL34, it is possible to reduce inflammation in humans and murine models of RA (28). Anti-CSF1R antibodies significantly down-regulate the production of IL6 and other inflammatory mediators in synovial explants of RA, and paw swelling and joint destruction in the CIA model. On the contrary, neutralisation of CSF1 or IL34 individually had no detectable effect on inflammatory gene expression in RA synovial tissue, supporting the hypothesis that these two cytokines were redundant in inducing differentiation and survival of tissue macrophage populations. These results introduce the hypothesis that interfering with the CSF1R binding to both CSF1 and IL34 is a possible novel therapeutic strategy for RA.

It is well known that the bone tissue balance is controlled by the “RANK/ RANKL/OPG/OPGL system”, tightly regulated by cytokines such as TNF-α IL1β, and IL6. These mediators seem to stimulate osteoclastogenesis through up regulation of receptor activator of nuclear factor κappa B ligand (RANKL) and down regulation of osteoprotegerin (OPG) in osteoblasts. Therefore, in RA, chronic inflammation leads to progressive joint destruction and bone resorption due to osteoclasts’ hyperactivity. Activated FLS, together with activated T and B cells, monocytes and macrophages, trigger the differentiation of osteoclasts via RANKL/RANK activation. During the process of bone loss in RA, the pro-inflammatory cytokine IL15 seems to be involved, inducing osteoclastogenesis in synergy with RANKL. IL15 does not influence osteoclast differentiation of mouse macrophage cell line RAW264.7 (RAW) by itself, whereas following co-stimulation of these cells with both IL15 and RANKL, a significant increase of genes characterising osteoclast differentiation and osteoclastogenesis were observed (29).

Innate immunity is probably involved in osteoclast differentiation also through TLRs. It is well known that TLRs are expressed in the RA joints and an increased serum and SF expression of endogenous TLR4-ligands (e.g. Tenascin-C, S100A8/A9, citrullinated fibrinogen (cFb) immune complexes) has been observed in patients with RA. However, their role in the pathogenesis of this disease is not fully understood. It has been recently demonstrated that joint inflammation and bone erosion in the CIA model is in part due to activation of TLR4 by various TLR4 ligands arising at different stages of disease. Some endogenous TLR4 ligands might be temporally up-regulated, and their increase seems to be associated with clinical and histological manifestation of CIA. Among endogenous TLR4 ligands, Tenascin-C was found to be up-regulated in the circulation of early phase of CIA mice. Furthermore, it positively correlated with the clinical score at day 56. S100A8/A9 was increased starting from day 28, peaking at day 42, and positively correlated with joint inflammation. Anti-cFb antibodies were increased during the late phase of CIA and positively correlated with both joint inflammation and cartilage damage (30). In addition, it was possible to prevent clinical and histological signs of arthritis by blocking the TLR4 activation in the early phase of CIA, using a neutralising monoclonal antibody against TLR4. In parallel it has been shown that Tenascin-C and cFb immune complexes promote osteoclast differentiation in vitro.

Taken together, these results demonstrate the direct involvement of TLR4 activation in the pathogenesis of CIA and in osteoclast differentiation, supporting the potential beneficial effect of targeting TLR4 in RA, especially in those patients with high levels of endogenous TLR4 ligands. The innate immune cells are closely linked to resident cells and they play an active role in the development of RA. In the synovial tissue, RA FLS play a major role in invasive joint destruction. During chronic inflammation, they are stimulated to migrate from the affected synovium to the healthy tissue, contributing to the spread of arthritis and destruction of distant joints. They invade the extracellular matrix (ECM) and secrete matrix metalloproteinases (MMPs), contributing to the destruction of the cartilage and bone tissues and leading to exacerbation of the joint damage. Activation of RA FLS may be due to the direct effect of the cytokine environment (e.g. TNF-α and IL6), the cell-to-cell contacts, or the activation of TLR2, TLR4, and TLR3. Following activation, these cells exert a pro-inflammatory activity by synthesising cytokines, chemokines, prostanoids, and nitric oxide (NO). Multiple signal transduction pathways might be involved, such as protein tyrosine phosphorylation, resulting from the balanced action of protein tyrosine kinases (PTKs) and phosphatases (PTPs). Recently, the transmembrane PTP RPTPκ, encoded by the PTPRK gene, has been found to be over-expressed in RA FLS compared with those of OA. Since PTPRK is a TGFβ-target gene, its over-expression in RA FLS results from an increased production of TGFβ. Therefore, RPTPκ promotes RA FLS invasiveness by regulating phosphorylation of the inhibitory Y527 of Src, enhancing in this way responsiveness to platelet-derived growth factor (PDGF), TNF
demonstrated that the PDGF receptor (PDGFR) expressed in RA FLS, promotes invadosome formation and matrix degradation through activation of PI3K/AKT pathway. PDGFR activation by phosphorylation is specifically increased in RA FLS and synovial tissues, and involves the upregulation of PDGF-B isoform induced by TGFβ. Indeed, among the PDGF isoforms, only the PDGFR-B was significantly elevated in RA FLS and was related to high-invasodome forming cells by activation of PI2K/AKT pathway (32).

Together with FLS and osteoclasts, mast cells play an important role in the inflammatory processes of RA. They are tissue dwelling cells with a relevant role in inflammation, fibrosis and angiogenesis. They role in the pathogenesis of RA has been widely investigated, but some aspects of their pathological behaviour in the development of the disease are still unclear. Recent data suggest that mast cells are involved in preclinical phase of arthritis, at least in the CIA model. In this animal model, mast cells seem to affect the adaptive immune response during the development of arthritis, by inducing a more powerful anti-inflammatory T cell response. Van der Velden et al., indeed, by studying the role of these cells during different phases of CIA in inducible mast cell knockout mice, have shown that depletion of mast cells in established arthritis did not affect clinical outcome, whereas a depletion induced during the preclinical phase resulted in a significant improvement of arthritis. This coincided with a decrease in circulating CD4+T cells and inflammatory monocytes, as well as of circulating levels of IL6 and IL17, but not of collagen-specific antibody. Furthermore, splenocytes from mast cell-depleted mice, stimulated with collagen type II, displayed reduction of IL17 and increased of IL10 production (33). Taken together, these results confirm that a close interaction of structural cells with innate immune cells takes place in RA. Therefore, targeting some signal transduction pathways in these cells might ameliorate some aspects of the disease.

**Adaptive immune response**

**T lymphocytes**

Besides the innate immune system, the adaptive counterpart plays a critical role in autoimmune diseases. Dysregulation of the adaptive immune response seems to be responsible for the development of RA. Up to now, an imbalance of effector and regulatory lymphocytes, leading to aberrant autoimmune response, is a hallmark of RA pathogenesis. As far as Th lymphocytes are concerned, recent data highlighted another aspect of the interaction between this T lymphocyte subset and cells of the innate immune system. Reynolds et al., described how monocytes from healthy donors can differentiate into a recently described subtype of DC, named inflammatory (INF) DC, following stimulation by granulocyte-monocyte colony stimulating factor (GM-CSF), mostly produced by SF CD4+ T cells and further increased after stimulation with IL12 (34). Besides CD4+/Th1 cells, several other T cell populations are active players in the pathogenesis of RA. The role of Th17 cells in the induction and maintenance of the inflammatory process is well established. However, the mechanisms leading to their aberrant expansion and activation are still not entirely clarified. IL23, a key cytokine for Th17 cell commitment, is increased in the circulation of RA patients (35). In addition, the recently discovered glycoprotein VSTM1-v2, over-expressed in RA PBMCs, is able to promote differentiation and activation of Th17 cells and is positively correlated with IL17 levels, disease activity, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in RA (36). Moreover, retinoic acid-related orphan receptor (ROR) γt expression in T cells, and therefore Th17 commitment, can be induced by a recently identified circulating bone-marrow-derived cell subset, named fibrocytes. These cells are precursors of FLS, are able to produce IL6, a key cytokine in the process of Th17 cell differentiation, and might be activated by Th17 cells. Of interest, fibrocyte proportion is higher in the PB of RA patients compared to the healthy subjects, suggesting an active role of these cells in RA pathogenesis (37). In addition to IL17, IL21 and IL22, through a complex network of intracellular signals, Th17 cells also produce RORγt, signal transducer and activator of STAT3 and IRF4. The protein inhibitor of activated STAT3 (PIAS3), a physiological inhibitor of STAT3, is down-regulated in RA PBMC compared to healthy subjects. Interestingly, miR-301a-3p, a PIAS3 regulating microRNA, is highly expressed in RA PBMC, accounting at least in part, for the decreased amount of PIAS3 and subsequent activation of Th17 cells (38). IL17 secretion can also be enhanced by a recently described Th cell subset, named Th9, through the secretion of IL9 (39), and stimulates T cell proliferation via the phosphatidylinositol 3-kinase (PI3K) pathway. IL17 can also hamper the chondrogenic potential of mesenchymal stem cells in the cartilage of RA, restored by blocking IL17 and TNF-α (40). An interesting and peculiar feature of Th17 cells is their plasticity towards different T cell phenotypes such as Th1 or T regulatory (Treg) cells. Indeed, the reduction of circulating Th17 cells observed in established RA may be the result of a shift of Th17 to either Th1 (41) or Treg cells. The latter hypothesis is supported by the observation of higher proportions of RORγt/FoxP3+ (forkhead box P3) double positive (DP)
T cells in RA PB. This observation further complicates the understanding of the role of Treg cells in RA pathogenesis. Recent findings demonstrated an elevation of the levels of the Treg stabilising protein progranulin, correlating with disease activity and inflammatory markers (42). However, the majority of studies performed in the recent year agree that Treg cells maintain a suppressive activity in RA patients (43, 44), suggesting a pivotal role of these cells in the regulation of the adaptive immunity during RA.

Another T cell subset involved in RA pathogenesis is represented by Thf, which are physiologically found in secondary lymphoid structures and germinal centres. Thf cells are characterised by surface expression of the CXCR3 chemokine receptor-5 (CXCR5) and are able to produce its ligand CXCL13, a chemokine that orchestrates ectopic lymphoid neogenesis. This chemokine can also be produced by a CD4+ cell subset, lacking its surface receptor CXCR5, that arises from naive T-cells following TGFβ stimulation (45). Besides CD4+ T lymphocytes, other T cell subsets, such as CD8α and CD4 CD8 cells were found to be involved in RA pathogenesis. A higher proportion of CD8α cells expressing the leucocyte immunoglobulin (Ig)-like receptor (LIR)-1, which is responsible for maintaining cytomegalovirus (CMV) infection in a latent state, has been detected in CMV+ RA patients compared to CMV+ subjects without RA, and its proportion correlated with the disease activity (46). Finally, CD4+CD8−γδT cells, known for their potential tolerogenic effect in pregnancy, have been linked to the frequently occurring spontaneous disease remission in pregnant women. This arises from the observation that in RA women the circulating TNF-α+ and IFNγ+γδT cells are down-regulated in pregnant compared to non-pregnant woman (47).

B Lymphocytes and autoantibodies

Moving to B lymphocytes, their peculiar pathogenic role in RA is to produce autoantibodies, mainly targeting citrullinated self proteins. The large amount of peptides produced by plasma cells leads to accumulation of misfolded proteins in rough endoplasmic reticulum (RER). One of the agents responsible for eliminating these proteins, growth arrest and DNA damage-inducible gene (GADD)34, is up-regulated in circulating B cells from RA patients. GADD34 correlates with circulating ACPA (48), which display glycosylated aminoacid residues in their variable domain and influence their binding to citrullinated antigens. Such post-translational modification, likely resulting from somatic hypermutation, represents an advantage that allows specific B cell subsets to be selected (49). ACPA can occur several years before RA onset and the site where PAD-mediated protein citrullination stimulate B cells to initiate ACPA production is still unknown. It has been recently demonstrated that RA FLS can produce four different isotypes of PAD (50). Among them, PAD2 is increased in cell-free SF and correlates with serum anti-CCP and disease activity (51).

By LC-MALDI-TOF, Wang et al. identified 83 over-citrullinated autoantigens in the SF from RA patients, but not in the control group. In addition, they demonstrated that the sites of citrullinated PADI2 and PADI4 were different in RA compared to the control group (52). ACPA also represent a bridge between genetic background, environmental factors and RA development. Gan et al. found a significant association of omega-3 fatty acid dietary intake with anti-CCP positivity in healthy subjects at increased risk of developing RA (FDRs) (53). In a similar way, Giant et al. characterised the structure and the localisation of citrullinated proteoglycan aggrecan (CitPG), the most abundant macromolecule of the articular cartilage (54). This antigen is found in both OA and RA cartilage, although its localisation is different in patients with RA compared to those with OA. A further work by Joshua et al. (55) better characterised the anti-citrullinated fibrinogen (cit-Fib) antibodies. They demonstrated that several cit-Fib peptides are targeted by autoantibodies in RA, but not in psoriatic arthritis (PsA) and ankylosing spondylitis (AS), implicating that these are more specific for RA. An association between cit-Fib autoantibodies and the R620W PTPN22 risk allele supports the hypothesis of an altered B cell regulation, such as auto-reactive B cells, which evade tolerance. Sialylation has been proposed as a feature of RA-associated IgG in humans and in mouse models of arthritis (56). In the CIA model, genetical blockade of sialylation in activated B cells leads to exacerbation of joint inflammation. On the other hand, artificial sialylation of anti-type II collagen antibodies, including ACPA, not only attenuates arthritogenic activity, but also suppresses the development of CIA in the antibody-injected mice. On the contrary, sialylation of other IgG does not prevent features of the disease in this animal model.

Recently, some novel mechanisms involved in the citrullination of ACPA have been described. For example, the ability of periodontal disease bacterium Porphyromonas gingivalis to induce citrullination via PAD production is thought to be relevant in the pathogenesis of RA. In support of these observations, an association between anti-RgpB IgG, antibodies directed towards a virulence factor of the pathogen, and the development of RA, has been reported (57), as well as the link between Porphyromonas gingivalis-derived ACPA and RA activity (58). The contribution of molecular mimicry between microbial antigens and autoantigens in RA is likely not limited to Porphyromonas gingivalis. HSP70-family binding Ig protein (BiP) is a candidate autoantigen in RA and share a substantial sequence with mycobacterial HSP70 (MycHSP70), mostly evident in the HLA-DR4 shared epitope binding sequences. MycHSP70 showed immunogenicity in RA in both humans and animal models (59). Some studies explored other promising RA-associated subsets of autoantibodies directed against carbamylated residues (anti-CarP), which recognise the homocitrullination of lysesins (60). However, the precise link between these new antibodies and ACPA is not yet fully understood. The introduction of anti-CarP antibodies allowed the researchers to identify new biomarkers, pathognomonic for the disease or re-
lated to clinical aspects of arthritis. The identification of an organ-associated antigen or a pathogen-derived epitope could elucidate the aetiology of the disease. A cross reactivity between the two groups of antibodies has been proposed. Turunen et al. showed that it is possible to induce a class of ureido-specific antibodies direct to both citrullinated and homocitrullinated antigens in rabbits immunised with type II collagen-related peptides, even in peptide sequences different from those used for the original immunisation (61).

Decades after the discovery of RF, its specific function in the pathogenesis of RA as well as its interplay with ACPA is still a matter of debate. An increased risk of RA development in healthy subjects with positivity of any of the three RF isotypes (IgM, IgG or IgA) in combination with any ACPA has been shown (62). However, the role of these antibodies in the mechanisms involved in RA pathogenesis is likely distinct, and this is supported by their different behaviour following pharmacological treatment. The recent study of Iannone et al. showed that anti-CCP levels may change during RA course, regardless of the biological drug used and the clinical response to the therapy (63).

Although B cells are considered part of the adaptive immune system, the B1 subtype does not show memory features and produces poly-reactive immunoglobulins, called natural antibodies (nIg) that are mainly of M class. They exert various physiological functions, but also show pro- and anti-inflammatory features. Interestingly, an increased amount of anti-galactosyl carbohydrate epitope (anti-Gal) nIgM (64) and a reduction in protective nIgM secretion induced by hypoxia-inducible factor (HIF)-1α were reported in RA patients (65). Finally, IgDs from RA patients seem to vigorously enhance proliferation of PBMC, activation of T and B cells and secretion of pro-inflammatory cytokines (66).

Besides the production of autoantibodies, more attention has been given to other functions of RA-derived B cells. An increase of B-cell lymphoma (Bcl)-2 protein expression seems to contribute to the reduction of B cells apoptosis, thus contributing to their expansion and pathogenic activity (67). An interesting in-vitro study revealed that B lymphocytes from healthy subjects can interact with OA FLS inducing the release of IL6, IL8 and MMP3, thereby inducing a RA-like destructive phenotype. On the other hand, FLS can hamper B cells, reducing their proliferation, secretion of immunoglobulins and production of pro-inflammatory cytokines (68). B cells can also express RANKL, which is enhanced in RA following B cell receptor (BCR) binding and IFNγ stimulation. Furthermore, co-cultures of B cells and monocytes showed a RANKL-dependent increase of osteoclastogenesis. These findings shed further light on the effect of B cells on bone damage (69, 70). This effect seems to be also exerted indirectly through secretion of different immunoglobulins. Lu et al. showed a positive correlation between serum antibody titre directed towards citrullinated-heat shock protein 60 (citrHSP60) and bone damage scores. Anti-citrHSP60 antibodies may be able to induce apoptosis in human osteoblast-generated sarcoma cell line (Saos-2) cells, after binding their citrullinated antigen expressed on Saos-2 cell membrane. These results specifically implicate the active role of ACPA and osteoblast surface-expressed citrHSP60 in the pathogenesis of RA (71).

Cytokines and chemokines

An array of soluble mediators contribute at different levels to the regulation of both innate and adaptive immune responses during RA. Among them, TNF-α is the main cytokine involved in the inflammatory response of RA, whereas cartilage and bone destruction is mostly directed by IL1. In addition to TNF-α and IL1, particular attention has been directed to IL6. This cytokine, produced by a variety of cells, mainly activated macrophages and FLS, has both pro- and anti-inflammatory properties. High levels of IL6 have been detected both in the circulation and SF of RA patients and a correlation of its levels with radiological joint destruction has been reported. Indeed, IL6 is probably involved in joint destruction by acting on neutrophils and on pre-osteoclasts in which promotes differentiation by either a RANKL-dependent or RANKL-independent mechanism. IL6 also acts in synergism with IL1β and TNF-α in producing vascular endothelial growth factor (VEGF), the main pro-angiogenic mediator, contributing to the maintenance of the synovitis. IL6 not only plays a pivotal role in regulating innate immunity, but it is also essential in modulating the adaptive immune response. This cytokine induces Th17 cell generation from naive T cells, by acting in synergism with TGFβ and inhibiting TGFβ-induced Treg (iTreg) differentiation. In normal conditions, Th17 and Treg cells remain in a dynamic balance just like the “Yin and Yang”. Recently, it has been shown that the “Yin Yang” transcription factor (YY1) may regulate the balance of Th17 cell and Treg cell via IL6 signalling pathway (72). This transcription factor, related to cancer development and progression, either activates or represses gene transcription, depending on the stimuli. An over-expression of YY1 in RA was found both in humans and in the animal model of CIA (DBA/1J mice). Its activity is mainly due to the binding to the promoter region of the IL6 gene, whereas IL6 and JAK/Stat signalling pathways were significantly inhibited by YY1 shRNA lentivirus (LV-YY1-shRNA) treatment. The blocking of the IL6 activity with LV-YY1-shRNA, down-regulates Th17 cells and ameliorates inflammation and disease progression in CIA mice as a consequence of the suppression of IL6 signalling pathway (73). Among the family of IL6/IL12 (IL2 superfamily), IL27 has been recently proposed as another mediator involved in RA pathogenesis. This cytokine is secreted by macrophages, epithelial cells and DC through IFNγ, and its receptor is present on B cells, CD4+ T cells, NK cells, mast cells and monocytes. IL27 might induce monocytes to release IL1β, TNF-α, IL18 and IL12 and it might contribute to the differentiation of Th1 in naive CD4+ T cells, which can secrete IL10 and IL17. Therefore, modulating the expression of IL10 and IL17, IL27 can promote Treg cell differentiation and exert broad inhibitory
effects on Th1, Th2 and Th17 cells. In addition, IL27 inhibits the formation of mature osteoclasts from pre-osteoclasts, by suppressing the expression of calcineurin-dependent 1 NFAT, which in turn down-regulates RANK signaling. Recently, an association of IL27 with disease activity and response to therapy has been hypothesised (74). In fact, an increase of IL27 levels in the circulation of RA patients positively correlates with the disease activity and seems to decrease following immunosuppressant treatment. Therefore, this cytokine could be a potential biomarker of disease activity and response to therapy in RA.

Among the IL1 family, IL33 plays a dual role both as an alarmin and a stimulus for Th2 response, exerting both positive and negative regulation of inflammatory processes, depending on the tissue environment. Increased levels of IL33 have been detected in serum, synovial tissue and cultured RA FLS, however its role in the pathogenesis of the disease is not fully elucidated. For example, exogenous IL33 could inhibit arthritis in CIA model, and mice deficient in the IL33 receptor ST2 show reduced susceptibility to arthritis, whereas the disease is not modified in IL33-deficient mice. Recent data have shown that the absence of IL33 does not affect the shift of T cells toward Th1, Th17 or Treg subpopulations (75). By evaluating two mouse models of chronic inflammatory diseases, CIA and imiquimod (IMQ)-induced psoriasis, Athari et al. showed that both arthritis and psoriasis developed independently of IL33 expression and does not affect the frequencies of Treg, Th1, and Th17 cells in both animal models.

Among IL12 family cytokines, the anti-inflammatory cytokine IL35 might also play a role in the angiogenic processes during RA. In both in-vitro and ex-vivo models, IL-35 might reduce synovial neo-vascularisation and inflammation by interfering on VEGF/Ang2 crosstalk. Its inhibitory effect on Ang2 expression and disruption of Ang2/Tie2 signal transduction seems to be responsible for down-regulation of angiogenesis and inflammation in the synovial tissue (76).

Finally, a number of other soluble mediators such as soluble adhesion molecules have been recently identified as potential modulators of RA. The soluble form of integrin CD18 (sCD18), secreted by leukocytes, is reduced in RA and is capable of competing with its membrane counterpart for the binding to ICAM-1 (77).

Interestingly, Boutet et al. investigated in RA the different isoforms of IL36 (IL-36α, IL-36β and IL-36γ), pro-inflammatory cytokines known to be involved in the pathogenesis of psoriasis. In CIA mice model and in the synovium of patients with RA, IL-36α, β, γ and their antagonists (IL-36Ra and IL-38) were all increased and correlated with the levels of IL1β, CCL3, CCL4 and M-CSF, but not with Th17 cytokines. However, they found differences in the ratio of these cytokines and their antagonists among patients with RA and other chronic conditions such as Crohn’s disease and psoriasis, and in some subgroups of RA patients. The rationale of these distinct expression profiles is currently debated, and further studies are required (78).

Conclusion

In the pathogenesis of RA several processes are involved. Dysregulation of the innate and adaptive immune responses occur at different stages of the disease. Innate and adaptive immune cells, soluble mediators, adhesion molecules and autoantibodies contribute to the development of inflammation and structural changes of joints and internal organs. The understanding of the mechanisms underlying the pathogenesis of RA will enable the development of novel and more specific disease-modifying therapies.

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