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The etiology of rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis is a heterogeneous disease, which can be, based on data combining genetic risk factors and autoantibodies, sub-classified into ACPA-positive and -negative RA. Presence of ACPA and RF as well as rising CRP-levels in some patients years before onset of clinical symptoms indicate that relevant immune responses for RA development are initiated very early. ACPA are highly specific for RA, whereas RF can also be found among healthy (elderly) individuals and patients with other autoimmune diseases or infection. The most important genetic risk factor for RA development, the shared epitope alleles, resides in the MHC class II region. Shared epitope alleles, however, only predispose to the development of ACPA-positive RA. Smoking is thus far the most important environmental risk factor associated with the development of RA. Studies on synovitis have shown the importance not only of adaptive but also of innate immune responses. In summary of the various results from immunological changes in blood and synovial tissue, the extension of the immune response from a diffuse myeloid to a lympho-myeloid inflammation appears to be associated with a more successful therapeutic response to biologics. With respect to advances in synovitis research, new targets for treatment against pathological subsets of immune cells or fibroblasts are already on the horizon. However, alternative strategies involving the microbiome may play an important role as well and research in this field is growing rapidly.

1. Introduction

Rheumatoid arthritis (RA) is a disease of unknown origin, which is characterized by inflammatory changes of the synovial tissue of joints, of cartilage and bone and, less frequently, of extra-articular sites. In recent years, it has become evident that RA arises based on genetic and epigenetic components, but also the environment must play an important role such as cigarette smoke, dust exposure and especially the microbiome that also represents an “internal” environment. There appears to be an important interplay between components of the adaptive immune system and the innate immune system. Abnormalities in the cellular and humoral immune response lead to the occurrence of autoantibodies, most notably rheumatoid factors (RF) and antibodies against post-translationally modified proteins (Anti-modified protein antibodies (AMPA) that comprise antibodies against various modifications such as citrullination (ACPA), carbamylation (aCarP) and acetylation (AAPA)) as well as the immigration of T and B-lymphocytes into the synovium. There is also an intense activation of the innate immune system with highly activated cells of the monocyte/macrophage system in the tissue sites involved. The clinical and histomorphological picture of RA is the result of distinct phenomena: Inflammation is reflected by joint pain, swelling and subsequent destruction of cartilage and bone, as well as systemic manifestations caused by metabolites of arachidonic acid and various inflammatory cytokines.

Synovial hyperplasia is a hallmark of RA and the main contributor to the formation of an invasive pannus. The observation of T cell accumulation in the synovium has generated the hypothesis of a T cell dependent inflammatory reaction to an unknown antigen. This assumption is supported by data derived from animal models, observations about disease remission in patients with AIDS and the improvement after treatment with co-stimulation modulators. In RA, the synovial lining, which normally comprises 1–3 cell layers, becomes remarkably thickened. This is due to an invasion of macrophage-like cells and the proliferation of resident synovial fibroblasts. The degree of synovial hyperplasia correlates with the severity of cartilage erosions resulting in inflammatory pannus formation, which attaches to and invades joint cartilage, while osteoclast activation leads to parallel bone destruction. Synoviocytes of this region secrete significant amounts of matrix degrading enzymes like collagenase, stromelysin, and gelatinase.

Although the processes initiating RA remain elusive, it can be stated that at the stage of fully expressed RA activated cells of macrophage and fibroblast origin dominate the destructive process. Efforts to elucidate the signal pathways that turn synovial cells into a highly aggressive pannus tissue have detected a whole network of interacting

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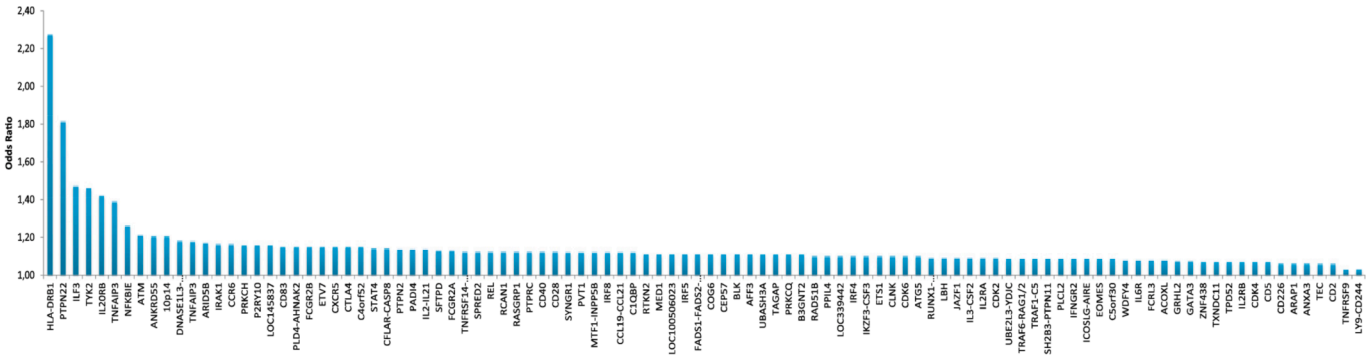

Fig. 1. Genetic risk loci (ranked by effect size) predisposing to the development of RA (Messemaker et al., Journal of Autoimmunity 64 (2015) 74–81).

cytokines. Factors like Interleukin-6 and Tumor Necrosis Factor-alpha are responsible for deregulating the balance between formation and degradation of cartilage and bone matrix within the joint. With these factors identified, new treatment regimens that selectively antagonize inflammatory cytokines have been applied very successfully.

2. Genetic susceptibility to RA

Genetic predisposition plays an important role in the development of RA. The concordance of the disease is only about 15% in identical twins but its overall heritability (a quantitative measure of the amount of variation in disease susceptibility that can be explained by genetic factors) has been estimated to reach 66% [1]. While this underlines the importance of genetic risk loci for RA, the degree of genetic risk in twins indicates that also environmental factors, presumably infectious events must play an additional decisive role. Extensive whole genome sequencing using various cohorts worldwide has revealed a large number of genetic risk loci that associate with RA (Fig. 1) [2]. Most of these are not unique to RA but rather reflect susceptibility alleles that associate with other autoimmune diseases as well, such as protein tyrosine phosphatase, non-receptor type 22 (PTPN22), for which a common single nucleotide polymorphism confers increased risk for the development of type 1 diabetes, RA, systemic lupus erythematosus, vitiligo and Graves' disease. This is also the case for genetic variants in the HLA class II region on chromosome 6, which harbors the strongest risk effect for many autoimmune diseases including RA. However, the HLA association in RA is by itself distinct and unique as the strongest genetic predisposition to RA associates with the inheritance of particular HLA-haplotypes. These are characterized by HLA-DRB1 alleles (especially DR4 and DR1) that specify molecules bearing a five amino acid “shared” motif (QKRAA, QRRAA or RRRAA in positions 70–74 of the DRB1 chain) encoding the positively charged P4 peptide-binding pocket, a motif that became known as the “shared epitope” (SE) [3,4]. Notably, HLA-DRB1 alleles carrying these SEs, and in fact many other of the genetic risk loci identified in other chromosomal regions, were found to only predispose to ACPA-positive RA, while non SE-bearing HLA alleles (in particular HLA-DR3 [5,6]) are risk variants for ACPA-negative disease. While this suggests that ACPA-positive and –negative disease are two distinct disease entities, it might also explain why the presence of HLA-DR4 is associated with a worse course of RA with severe joint destruction, particularly when both chromosomes code for this molecule (“double dose” of the gene). To date, it remains still incompletely understood how the SE-bearing HLA-DRB1 haplotypes confer risk to ACPA-positive RA. Mechanistically, the HLA-DR predisposition suggests the ability of the SE-bearing HLA-DR molecules to present self-peptides that lead to a tolerized CD4 T cell repertoire. These peptides would likely bear a negative or neutral charge at P4, enabling them to bind to the positively charged SE molecule P4 pocket, while peptides containing positively charged arginine or lysine at P4 would be unlikely to bind with measurable affinity because of the repulsion of similar charges. Consequently, one hypothesis of the effects of citrullination is that modified peptides are created that can bind (more effectively) to the MHC class II encoded susceptibility molecules. By altering the property of peptides containing positively charged arginine or lysine at P4, these would bind to SE molecules, based on the modification involving the conversion of the arginine and lysine residues to the neutral-polar citrulline [7]. Indeed, several studies have demonstrated that citrullinated peptides are accommodated in the peptide-binding pocket more efficiently than their native counterparts [8,9]. However, more recent work refined the HLA association and revealed that three amino acid positions in the beta chain of HLA-DR (11, 71 and 74) and one in HLA-B and one in the beta chain of HLA-DPB1 (both at position 9) are responsible for the association with RA [10]. Also, detailed binding studies have shown that the enhanced presentation of citrullinated peptides appears not to be restricted only to SE-motif bearing HLA-molecules, as differential peptide binding was also found for certain HLA-DQ molecules and pockets in HLA-DRB1 SE-bearing molecules other than P4 [11]. Hence, the molecular basis for the RA risk effect conferred by HLA-SE molecules and its contribution to the loss of tolerance to citrullinated antigens may be more complex than anticipated in the original SE hypothesis. Therefore, central unanswered questions in the pathogenesis of RA remain, in particular:

- Which relevant peptides are presented by the molecules encoded by these susceptibility alleles,
- how does the T cell response in RA relate to the recognition of these peptides and
- how do these events contribute to the disease origin?

A second potential effect of citrullination is the creation of neo-peptides as a consequence of altering peptide processing through affecting molecular conformation by interruption of H bonding to arginine and lysine, respectively, and would result in the generation and MHC binding of novel peptides, not present during formation and initial tolerization of the CD4 T cell repertoire [12]. One can envision that during the clinically asymptomatic (preclinical) phase of autoantibody seropositivity (see below), the continuous generation of additional modified self-proteins and their interaction with locally produced RF, ACPA and anti-CarbP antibodies reinforces T cell activation. This could lead to the generation of an increasing number of modified peptides that are recognized (‘epitope spreading’) and would foster selection of cognate T cells with greater affinity for self-peptides. This would ultimately lead to clinical disease.

3. Conceptual understanding of RA development

As stated above, genetics indicate that the clinical phenotype of RA segregates in at least two different disease entities for which the

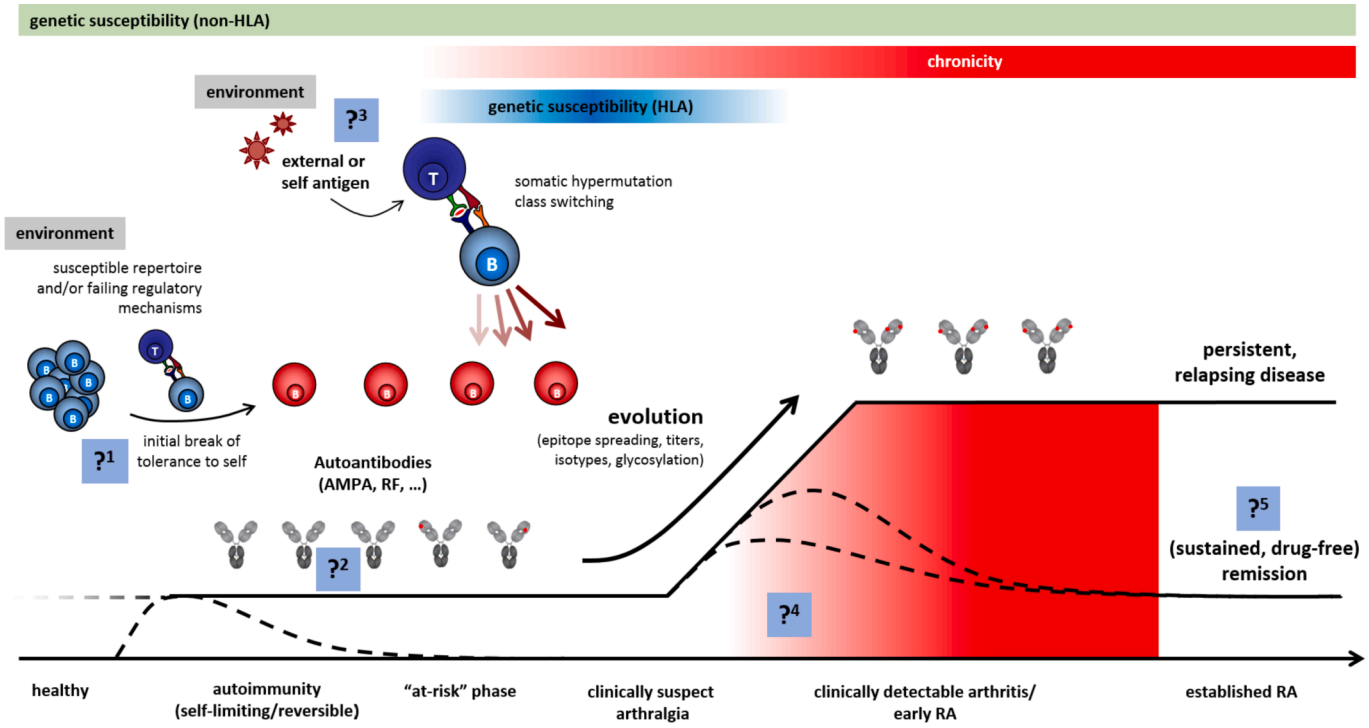

Fig. 2. Conceptual model depicting factors involved in the development of autoantibody-positive RA
Schematic depiction of immunological events in the preceding phase of autoantibody positive RA, combined with several questions that remain unanswered to date. Moving from left to right, genetic predisposition and environmental factors likely govern the earliest break of self-tolerance, which might occur under the influence of T cells and/or innate triggers on the basis of a genetically susceptible B cell repertoire and/or failing mechanisms/checkpoints of tolerance control. In the model depicted, these early events lead to systemic autoimmunity (i.e. the detectable presence of autoantibodies such as AMPA or RF) which by itself is initially reversible and self-limiting. Under the influence of repetitive triggering and/or other unknown factors, transient systemic autoimmunity may become persistent but can remain clinically quiescent for many years. The presence of glycans in the ACPA-IgG variable domain (depicted as red dots) at this stage confers risk for future development of RA; such glycans are highly abundant in established disease. Prior to the development of arthritis, the AMPA B cell response evolves and broadens, as evidenced by rising serum titres, epitope spreading, extended isotype usage and changes in antibody Fc glycosylation. HLA-SE positivity strongly predisposes to this step (rather than to the initial break of tolerance), indicating that the pre-disease evolution of the AMPA response is likely governed by T cells. How these processes eventually induce inflammation in joints remains unclear.
Unanswered questions:

- ?[1]: Which factors/antigens (self and/or foreign) and modifications induce the initial break of tolerance to self-antigens?
- ?[2]: Which factors induce persistence of systemic autoimmunity?
- ?[3]: Which antigens (self and/or foreign) drive the evolution of the AMPA response, i.e. the transition from systemic autoimmunity to autoimmune disease?
- ?[4]: Do the immunological processes depicted eventually induce synovial inflammation, and how?
- ?[5]: Does the AMPA response and its characteristics drive disease persistence/chronicity, and are the processes underlying the AMPA response reversible in established disease?

presence of antibodies directed against citrullinated protein antigens (ACPA) serves as testable biomarker and surrogate of the underlying immunological processes [5,13–16]. This observation has been instrumental to intensive research into the etiology of ACPA-positive disease. Together with the recognition that ACPA are present in the seemingly healthy, symptom-free pre-disease phase of RA [17,18], a detailed analysis of the risk effects mediated by ACPA in this phase and in the phase of clinically suspect arthralgia (CSA) [19–21], and a detailed refinement of the contribution of HLA-SE alleles to disease [22,23], this has led to a conceptual framework of RA development which now has translatable clinical implications (Fig. 2). In fact, both clinical and immunological descriptive definitions of different disease phases have allowed for the development of prediction models for pre-disease at-risk individuals and the conduct of first clinical trials that aim at disease prevention in the phase of pre-clinical RA [24,25]. The etiology of ACPA-negative disease and of its developmental stages, however, is still poorly studied and less well understood. Also here, we will focus on our current understanding of the ACPA response, on the recent description of autoantibodies against additional posttranslational protein modifications, and on potential implications of these responses in disease pathogenesis.

3.1. Autoimmunity and autoimmune disease - different phases in RA development

In many autoimmune diseases, autoimmunity (as defined by the presence of autoantibodies that more or less specifically associate with disease) precedes the development of autoimmune disease. In many cases, this observation is based on retrospective studies starting from cohorts of patients with established disease [17,18,26]. Hence, for a long time it was (and in many cases still is) incompletely understood whether the presence of disease-specific autoantibodies in individuals without symptoms inevitably leads to disease at some point in time, or whether autoimmunity is reversible without a pre-defined path to chronic disease. For RA, population- and family-based studies in twins and in first-degree relatives of patients have shed light on these questions [22,23,27–29]. ACPA but also RF and antibodies to carbamylated proteins (aCarP), are clearly detectable and frequently present in the phase preceding RA. External triggers such as smoking might be involved in an initial break of tolerance and have been identified as risk factors for the development of these autoantibodies [23,30,31]. Also, the prevalence of ACPA in healthy, first-degree relatives of RA patients increases with age, in particular in postmenopausal women [29]. If

present at diagnosis (i.e. in the presence of clinically detectable arthritis), ACPA associate with disease severity, negatively impact on the chance to achieve sustained drug-free remission, and hence confer risk for disease chronicity [21]. Also, if present in the phase of clinically suspect arthralgia (CSA), a recently defined risk-phase of imminent RA in which progression to inflammatory arthritis within two years is around 20%, ACPA strongly confer risk for progression with a positive predictive value of 63% [19,20,32]. Hence, it is conceivable that ACPA and/or the underlying autoimmune response, for which ACPA may be surrogate markers, are directly involved in development and maintenance of chronic arthritis.

In the general population, however, this might be different as the prevalence of ACPA exceeds the prevalence of ACPA-positive RA [23,28]. Also, in healthy first-degree relatives of patients, ACPA-positivity is comparatively frequent but not a stable trait [27]. In fact, in a recent study conducted in healthy Indigenous North Americans, reversion of ACPA-positivity to a seronegative state was frequent, despite this being a genetically susceptible population with a high incidence rate of seropositive RA [27]. Even if both ACPA and RF were present, seroreversion was frequently observed, with a likelihood of ACPA/RF double positivity reverting to seronegativity after five years of more than 30%. Hence, autoimmunity as defined by the presence of ACPA or RF is by itself a potentially transient, reversible state that likely requires additional triggers to develop into a sustained, chronic autoimmune response that eventually associates with disease precipitation.

3.2. Transition from autoimmunity to autoimmune disease

Given these considerations, it is an intriguing and ongoing challenge to identify, define and understand the mechanisms that drive the transition from (potentially reversible) autoimmunity to (potentially irreversible, chronic) autoimmune disease. In this context, particular importance belongs to the observation that the HLA-SE alleles confer risk to ACPA-positive RA rather than ACPA-positive autoimmunity in the absence of inflammatory arthritis (Fig. 2). In fact, two independent studies demonstrated the quasi absence of an HLA-SE risk effect on the presence of ACPA in individuals without RA [22,23]. This suggests that the transition from autoimmunity to autoimmune disease requires HLA class II-mediated immunological effects, which implicates a role for T cells in this phase, as noted above. To avoid confusion, the initial break of tolerance to citrullinated antigens, which leads to the initial generation of ACPA might well be controlled by T cells as well, as immunoglobulin (Ig) class-switching to IgG occurs already in the earliest, non-HLA-SE associated phase. However, this has not been formally shown and remains a matter of speculation, in particular as T cell help to B cells is not an absolute pre-requisite for Ig class-switch recombination [33]. In addition, the B cell receptor (BCR) repertoire of ACPA-expressing B cells in this phase has not yet been defined. On the other hand, the HLA-SE association with ACPA-positive RA indicates that a second, potentially different T cell response might be required to initiate the transition towards an ACPA-response with relevance for disease. To date, the nature of this response, the antigens recognized and the dynamics by which it might impact on a pre-existing ACPA B cell response, remain elusive.

Nonetheless, the concept of T cell help as driver of the transition phase to disease is supported by the observation that the ACPA response clearly expands prior to the development of inflammatory arthritis, with phenotypic changes that are compatible with a T cell driven process [34]. For example, the epitope recognition pattern of ACPA in serum expands prior to the onset of arthritis, with newly recognized epitopes recruited to the repertoire [35,36]; serum levels of IgG-ACPA rise and ACPA with additional Ig isotypes are expressed [37,38]. In addition, several interesting changes in the glycosylation of secreted ACPA-IgG have been detected in the pre-clinical phase of RA [39–41]. With reference to the ACPA-IgG Fc tail, a shift towards a Fc-glycosylation profile associated with pro-inflammatory effector functions has been noted shortly before the onset of arthritis, a finding which in mice could be mechanistically linked to the impact of Th17 cells on, in this case, collagen type II-specific B cells [42]. In experimental arthritis, these Th17 cells accumulated in germinal centers during the pre-phase of arthritis and suppressed the expression of sialyltransferases in plasmablasts, leading to the expression of IgG molecules with Fc-glycans lacking sialic acid, a phenotype that is closely linked to enhanced triggering of ITAM-bearing, i.e. activating Fc receptors [43,44]. While these observations suggest that a T cell-mediated effect on the ACPA B cell response could occur in relatively close temporal relationship with the onset of inflammatory arthritis, the dynamics of the transition phase in human RA are essentially unclear. In this respect, it is intriguing that ACPA-IgG molecules in the phase of established RA were found to carry not only Fc tail glycans but also abundant glycans in the antibody variable (V-)domain [45]. These glycans resemble those found on the Fc tail, as both are linked to the protein backbone via asparagine (N) residues and, hence, belong to the family of *N*-glycans [46]. However, in contrast to the ACPA Fc glycans, glycans found in ACPA-IgG V-domains are highly galactosylated and sialylated. Also, these glycans are found on almost all (> 90%) secreted ACPA-IgG molecules, while this is only the case for 15–25% of conventional serum IgG molecules [47]. In fact, between two and up to six *N*-linked glycans were detected on individual ACPA-IgG and located in either the heavy chain, light chain or in both [48,49].

So far, no other autoantibodies have been described that carry V-domain *N*-glycans at similar frequency, raising the question whether these glycans could confer effector functions to ACPA-IgG that could be relevant to disease. Currently, this question remains to be answered, in particular for secreted ACPA-IgG. However, the abundant presence of V-domain glycans is informative with respect to the ACPA B cell response and its development, as *N*-glycosylation requires the presence of a defined consensus sequence in the protein backbone of the V-domain. Such *N*-glycosylation consensus sequences consist of a tripeptide sequon (Asn–X–Ser/Thr (where X is any amino acid except proline)) that is encoded by only very few V-region genes of the germline repertoire [50]. Thus, B cells expressing V-domain glycosylated B cell receptors (BCRs) have to either express one of these V-regions (which would lead to a highly restricted, antigen-specific repertoire) or generate de-novo *N*-glycosylation sites in the V-region, which is possible during somatic hypermutation, a process usually initiated during germinal center responses under the influence of helper T cells [51]. In fact, detailed sequence analysis of citrullinated antigen-specific BCRs demonstrated the presence of *N*-glycosylation sites in almost 90% of ACPA-IgG V-regions, which by far exceeds the frequency of such sites in a healthy repertoire [48]. Importantly, however, all detected sites were indeed generated by mutation, making it highly likely that ACPA-expressing B cells receive T cell help during their development and/or expansion. At the same time, this raises the intriguing question whether ACPA-IgG in the earliest phase of asymptomatic autoimmunity also display V-region *N*-glycosylation to a similar degree, or whether the occurrence of this feature could serve as a marker signaling T cell help in the transition phase to autoimmune disease. So far, this question has been investigated in two studies, which both showed that ACPA-IgG V-region glycosylation can already be found in the phase of pre-clinical RA [40,41]. Notably, however, the degree of ACPA V-domain glycosylation in ACPA-positive, first-degree healthy relatives of Indigenous North American RA patients was significantly lower than in patients with established RA [41]. In fact, a high degree of V-domain glycosylation in the pre-disease phase was a strong predictor of transition to inflammatory arthritis.

Moreover, the second study demonstrated an association between the presence of HLA-SE alleles and the degree of ACPA-IgG V-domain glycosylation [40]. Together, these observations fit well with the concept of T cell help as trigger of maturation of a pre-existing ACPA B cell response towards a stage in which it might impact on or drive disease precipitation (Fig. 2). As a high degree of V-domain glycosylation was already present in some individuals years before the onset of arthritis,

however, it is likely that the T cell-induced maturation of the ACPA B cell response is not a single event but a process that rather requires multiple rounds of repetitive T cell help, possibly driven by different antigens and/or different T cells that can extend over a period of years. Also, it is conceivable that additional, so far unidentified factors may be involved that influence the dynamics of this process.

3.3. The initiation phase of inflammatory synovitis

The aspects discussed above delineate our current understanding of the immunological processes that lead up to the precipitation of disease in patients harboring an ACPA response. How these processes eventually lead to synovial inflammation of joints and the erosion of bone, however, is largely unclear. As will be discussed below, the hypothesis that ACPA themselves as functional autoantibodies are instrumental to early inflammatory changes and bone loss has been a matter of debate and still requires detailed investigations. However, circumstantial evidence suggests that B cells, whether auto-reactive or not, are relevant to the initiation of synovial inflammation and the changes of bone observed during this process. A one course therapeutic depletion of B cells with rituximab (a monoclonal antibody targeting the CD20 molecule expressed by immature, naïve and memory B cells) in ACPA- and/or RF-positive at-risk individuals with arthralgia, for example, caused some delay in the transition to inflammatory arthritis [24]. Moreover, a subset of at-risk individuals was found to harbor dominant B cell clones (potentially plasmablasts) in the circulation in the pre-arthritis phase, a feature that was strongly associated with the progression to RA [52]. Intriguingly, these clones disappeared from the circulation in the phase of arthritis and became detectable in the synovial tissue, raising the possibility that early migration of plasmablasts towards the synovial compartment could be involved in initiating synovial inflammation. In fact, early studies already demonstrated the presence of related B cell clones in different joints in patients with established disease [53]. Notably, the clonal expansion of B cells in the pre-arthritic phase was observed irrespective of the presence of ACPA, and the specificity of the expanded clones remains so far unknown. Nonetheless, ACPA-expressing B and plasmacells have been detected in the synovial compartment [54], and the concept of clonally expanding, presumably activated B cells as drivers of the transition to arthritis would fit well with the aforementioned data. Hence, this concept warrants more detailed investigation, in particular with regard to the its amenability for therapeutic intervention.

From a more structural point of view, histological studies in mice and joint biopsy studies in human RF- and/or ACPA-positive at-risk individuals indicate that synovial tissue remains normal until shortly (a time-span of days in mice) before the onset of macroscopic/clinically detectable arthritis [55,56]. In animal models, activation of macrophage-like synoviocytes and an infiltration of $CD4^+$ T cells were noted as the earliest histological events [57]. In addition, infiltration of tendon sheaths by inflammatory cells and the formation of osteoclasts in close proximity to the inflamed tendons were noted in the pre-clinical phase of experimental arthritis [58]. In humans, imaging studies in patients with CSA demonstrated the presence of subclinical inflammation in the form of varying degrees of synovitis, bone marrow edema and/or tenosynovitis in the pre-arthritic phase of RA [59,60]. This subclinical MRI inflammation associated with clinical arthritis development and preceded arthritis with a few weeks to months [19].

Notably, such general features of sub-clinical inflammation were not restricted to the autoantibody-positive group of individuals but could also be found in the ACPA-negative subgroup. In addition, MRI subclinical inflammation was also associated with the development of joint erosions [61,62]. In fact, structural changes in bone architecture could already be detected in ACPA-positive healthy individuals, together with loss of bone mineral density [63]. Also in early arthritis, loss of bone mineral density has been noted as a remarkable feature of the ACPA-positive subset, and mechanistic studies suggest that activated B cells can promote this process by the secretion of RANKL [64–66].

So far, however, the sequence of events with regard to the development of sub-clinical inflammation in the different joint-related structures (bone, synovium, tendons), that is whether inflammation starts within the joint or rather from the 'outside', is still not entirely clear [67]. In a cross-sectional analysis of ACPA-positive individuals without arthritis, MRI-detected tenosynovitis was the most prevalent feature, followed by synovitis and, less frequently, bone marrow edema [68]. In longitudinal imaging analyses employing MRI in individuals with CSA, tenosynovitis and synovitis were comparably found as the earliest features, while bone marrow edema (a surrogate of osteitis containing lymphocytic infiltrates) seemed to occur as a secondary event [68]. Whether this is the case during the developmental stages of ACPA-positive and ACPA-negative RA needs more detailed investigation, in particular as ACPA-positive individuals showed higher osteitis scores already at presentation compared to their ACPA-negative counterparts. Regardless of the temporal relationship, however, several studies demonstrated that bone marrow edema (osteitis) is strongly associated with the development of bone erosions in early, undifferentiated arthritis, early RA but also in the established phase of disease, independent of synovitis [69–72].

Taken together, the (immunological) mechanisms underlying the onset of synovial inflammation and osteitis need to be further delineated as targeted intervention in this phase holds the promise of preventing disease. In contrast to the phase of ACPA positivity in health in which the conversion rate to arthritis is low so that the conduct of clinical trials is difficult, the phase of clinically suspect arthralgia is now well defined and conduct of trials is feasible [25,32,73]). The choice of intervention, however, needs to move from rather empirical approaches to targeted intervention based on detailed knowledge of the underlying disease processes. Whether chronicity can (still) be prevented at this stage remains to be shown, but as (very) early intervention in RA increases the chance to reach drug-free sustained remission (a proxy for cure), the premise is favorable.

4. Anti modified protein antibodies in RA

4.1. Characteristics and effector functions of the ACPA response

As discussed above, the ACPA immune response plays a prominent role in the different developmental stages of RA and is closely linked to both genetic background and course of disease. Hence, intensive research efforts have focused on the characteristics of this response and its potential involvement in disease-specific pathogenic processes [21]. As ACPA themselves are detectable for years in the absence of inflammation, and as these antibodies persist in established RA and in patients in drug-free sustained remission it has been postulated that the quality of the ACPA-response rather than its quantity could determine its involvement in the inflammatory response observed in RA. Hence, numerous qualitative aspects of ACPA have been assessed, these include:

- the fine-specificity (i.e. epitope recognition) pattern of the response [74,75],
- the degree and type of ACPA glycosylation both in the Fc region and in the variable domain [45,76],
- its isotype usage [37,38,77,78],
- avidity [79],
- its potential to activate the complement cascade [80],
- and more recently also phenotypic features of ACPA-expressing B cells [81,82].

Next to the evolution of the response in the phase preceding arthritis that have been discussed above, these studies have highlighted the polyclonal nature of the response and the extensive cross-reactivity of ACPA towards different citrullinated peptide and protein antigens. In

fact, numerous citrullinated antigens have been identified that are recognized by patient-derived ACPA, including non-disease related proteins such as citrullinated myelin-basic protein (MBP), which is primarily expressed in the brain. For diagnostic use, different assays have been developed that employ synthetic cyclic citrullinated peptides (CCP) or somatically mutated versions of citrullinated vimentin (MCV) that each have proven to capture a broad range of serum ACPA with overall high specificity and high sensitivity [83,84]. More recently, the structural basis for this cross-reactivity has been elucidated by the generation of crystal structures of monoclonal ACPA [85]. These structures revealed that the citrulline residue is recognized by a non-polar conserved pocket in the antibody paratope, with characteristics of the citrulline flanking residues and the flexibility of the peptide allowing for conformational rearrangements determining cross-reactivity. Hence, the distinct structural accommodation of citrulline in the antigen-binding pocket of ACPA allows for the extensive cross-reactivity observed in peptide and protein arrays [86]. Notably, the degree of cross-reactivity varies largely between different monoclonal ACPA.

Interestingly, this cross-reactivity might also explain why the ACPA-response fails to develop into a high-affinity response such as, for example, anti-vaccine responses [79]. In fact, ACPA-expressing B cells undergo extensive somatic hypermutation [48,87], a process that in conventional immune responses is paralleled by affinity maturation, yet secreted ACPA remain of remarkably low avidity. Possibly, the ability of ACPA-expressing B cells to respond to different citrullinated antigens and, hence, the ability to receive help from different T cells, together with the abundant presence of antigen that alleviates the selective pressure of competition, could result in this overall low avidity response. To what extent also the V-domain glycans influence ACPA avidity is subject of ongoing studies.

Despite the broad cross-reactivity of ACPA and the expansion of the epitope recognition repertoire in the phase preceding arthritis, the fine specificity profile of individual patients is neither uniform nor identical and not necessarily stable [36,37,75,88–91]. This has led to the interesting hypothesis that distinct ACPA recognizing defined citrullinated antigens, or on broader terms that distinct ACPA recognition patterns could associate with disease phenotypes and defined, ACPA-mediated effector functions. In fact, variations in ACPA recognition patterns have been detected in different populations [92]. Also, certain recognition patterns were found to associate with differential responses to treatment [93,94]. Furthermore, responses to defined citrullinated antigens such as, for example, MCV have shown associations between serum titers and disease activity [83]. These findings have fuelled the hypothesis that ACPA directed against particular citrullinated antigens, for example against citrullinated vimentin could possibly serve as functional autoantibodies and induce defined pathogenic effector functions whereas those directed against other citrullinated targets may exhibit different or no effects. Several studies favoring this concept suggest that polyclonal ACPA bind to citrullinated antigens expressed on effector cells such as osteoclasts and fibroblasts [95–98]. In addition, also monoclonal ACPA demonstrated binding to cellular surfaces, notably via their Fab-fragments, with different monoclonal antibodies exhibiting different binding patterns [95,99]. Finally, the expression of citrullinated antigens, which for some cells required priming, coincided with ACPA binding. Hence, it indeed seems possible that ACPA act as functional autoantibodies. However, the (presumably citrullinated) antigen(s) recognized on either cell type have not been identified. Therefore, the cellular effects observed as a function of ACPA binding to such antigens, namely osteoclast activation and fibroblast migration, remain still controversial to date and will require independent replication and, eventually, receptor identification [100–104].

While this is pending, an alternative (non-exclusive) scenario is that the epitope recognition pattern could primarily reflect the maturation/expansion state of the ACPA B cell response while the identity of the citrullinated antigens that are part of the pattern might be less relevant. This would be in line with the observation that individual ACPA-fine-specificities, amongst which some targeting citrullinated antigens that have been found in joints, did not influence the degree of joint destruction and its progression over time [105]. Rather, in patients with early onset, untreated RA, a broad epitope recognition profile was found to associate with early treatment response but lowered the chance to achieve drug-free remission [106]. Also, a broad reactivity of the response in patients in sustained remission was associated with relapse of arthritis upon treatment discontinuation [107]. These observations were made with regard to the number of citrullinated epitopes recognized as a measure of the breadth of the immune response, but also with regard to the recognition of other posttranslational modifications and the presence or absence of rheumatoid factor. Hence, a broad auto-reactivity pattern as a result of a broadly activated B cell response seems relevant to the disease, which might also be reflected by the enhanced response to rituximab treatment observed in the autoantibody positive subgroup of patients [108]. Nonetheless, either scenario does not exclude that binding of ACPA to citrullinated antigens favors inflammation. In fact, irrespective of the functional effects that binding of ACPA might induce in cells, it is likely that ACPA-binding to citrullinated antigens leads to immune complex (IC) formation, in particular in localized compartments such as the joints [109–111]. Such ICs can strongly induce and enhance inflammatory responses by binding to activating Fc receptors, a mechanism possibly boosted by RF [110,112–116]. Also, pain induction was recently linked in murine studies to the FcR-mediated stimulation of neurons, as was the induction of osteoclastogenesis [117–119].

Taken together, many pieces of evidence support an inflammation-enhancing role for ACPA and for the ACPA B cell response in the pathogenesis of RA. The exact mechanisms underlying these effects still require further investigation.

4.2. Anti-carbamylated and anti-acetylated protein antibodies

The observation that citrulline is the common determinant of those RA-specific autoantibodies that were already described in the early 1960′s (termed anti-perinuclear factor at the time) substantially impacted on the refinement of the etiology of RA [88,120–123]. The more recent observation that RA patients also harbor autoantibodies directed against other posttranslational modifications (PTMs) might be considered of similar impact, as the nature of these PTM responses and their interrelation could be of substantial relevance to understanding the emergence of ACPA [124].

In contrast to citrullination, which is a modification of arginine residues, homocitrullination (carbamylation) is the result of the modification of lysine residues. Hence, although structurally similar to citrulline, homocitrulline residues are located at different positions in the protein, have different neighboring amino acids and are, by definition, different antigens. Antibodies targeting this PTM, termed anti-carbamylated protein (anti-CarP) antibodies, have been detected in around 45% of RA patients. Interestingly, anti-CarP antibodies were also found in 16–20% of ACPA-negative patients, in whom their presence associates with severe radiographic damage progression. Comparable to ACPA, anti-CarP antibodies are detectable years before the onset of inflammatory arthritis and associate with disease development. Also for this immune response, epitope spreading is observed in the wake of disease precipitation [125,126], but in contrast to ACPA, the presence of anti-CarP antibodies does not associate with the HLA SE alleles. Notably, anti-CarP antibodies are readily inducible in mice upon immunization with carbamylated antigens, which for ACPA is not as readily the case [127]. By itself, however, the induction of anti-CarP antibodies in mice does not induce arthritis. Nonetheless, the appearance of anti-CarP antibodies was also observed in the collagen-induced arthritis model, a model in which arthritis is induced by immunization with collagen type II and an adjuvant. In this model, the appearance of anti-CarP antibodies preceded arthritis onset and enhanced arthritis severity [128], although it was not a prerequisite for the development

of arthritis. Interestingly, anti-CarP antibodies were inducible upon immunization with type II collagen in complete Freund's adjuvant, but also when mice were administered complete Freund's adjuvant only, indicating that non-specific inflammatory triggers (such as infections) might be sufficient to breach tolerance to this modification. As for ACPA, it is possible that these inflammation-induced responses are normally transient and that additional triggers and/or genetic background are required to induce a chronic response that associates with disease. In this context, it is intriguing that there is considerable cross-reactivity between human ACPA and anti-carP antibodies. Isolation of ACPA by affinity purification using citrullinated antigens, for example, yielded a pool of antibodies that also showed reactivity to carbamylated antigens [129]. Vice versa, isolation of anti-carP antibodies by virtue of their affinity to carbamylated antigens also yielded ACPA. Hence, this observation considerably broadens the antigenic repertoire that could stimulate B cells reactive with PTM antigens.

An additional layer of complexity is added by the observation that RA patients harbor, next to ACPA and anti-CarP antibodies, auto-reactive B cell responses against additional PTMs, such as acetylation and potentially others. As for carbamylation, acetylation occurs on lysine residues, and anti-acetyl-lysine antibodies have been detected in 30–40% of RA patients [130]. Cross-reactivity of ACPA and anti-CarP antibodies extends to this PTM, as acetyl-lysine reactivity was also observed in the citrulline and homocitrulline affinity-purified antibody pools described above, both on the protein and individual peptide level [131]. Hence, based on these data that employed polyclonal antibody purifications, there appears to be broad cross-reactivity not only towards different antigens carrying one type of PTM, but also towards antigens carrying different PTMs that are structurally distinct and located in different positions of a given protein or peptide. To what extend this cross-reactivity pattern is also observed for monoclonal antibodies is a relevant question, which is currently being addressed by different groups. Nonetheless, based on the current data, the concept emerges that ACPA are part of a broader spectrum of autoantibodies that target PTMs and that can collectively be termed anti-modified protein antibodies (AMPA).

4.3. Generation of AMPA

So far, it remains unknown how, where and why tolerance to PTM-antigens is broken. Initially, research has focused on triggers of ACPA responses, such as citrullinated antigens expressed by pathogens [132]. In addition, citrullination of proteins and early lymphocyte infiltration was observed in lung-tissue of ACPA-positive RA patients [133–135]. However, the observation that ACPA are cross-reactive to different PTMs indicates that PTM-reactive B cells can be triggered by different PTMs and, maybe more importantly, can present these antigens to, and receive help from, various PTM-reactive T cells. In fact, different human AMPA were demonstrated to recognize modified self- and non-self-proteins [136], opening the possibility that non-auto-reactive T cells recognizing modified foreign antigens can provide help to B cells that, by cross-reacting to both foreign and self PTM-proteins, generate autoimmunity [124].

In mice, it was indeed possible to induce an anti-CarP antibody response to murine self-proteins upon immunization with carbamylated proteins of non-self origin. Furthermore, immunization of mice with carbamylated foreign proteins induced not only an anti-CarP antibody response to both foreign antigens and autoantigens, but also antibodies directed against acetyl-lysine modified proteins [131]. As ACPA were not convincingly found in these mice, it remains to be determined whether cross-reactivity to citrullinated antigens can also be induced via this pathway. However, the cross-reactivity of affinity-purified ACPA from human serum to both carbamylated and acetyl-lysine modified proteins strongly suggests that similar mechanisms might be at play in the human situation. Conceptually, these data indicate that B cell autoimmunity to citrullinated antigens emerges as part of autoimmunity to PTMs without the need for auto-reactive T cells. While the identity of these cells and of the antigens they recognize together with their localization remains elusive, the cross-reactivity to foreign antigens allows for the hypothesis that these might be of environmental origin.

5. The role of rheumatoid factors in RA

In 1937 Erik Waaler described an antibody in the serum of patients with RA that agglutinated sheep red blood cells. In 1949, H. M. Rose re-described this assay, and the subsequently developed Waaler–Rose test used sensitized sheep erythrocytes to detect rheumatoid factors, but modern tests use nephelometry or, ideally, an ELISA system, which can detect rheumatoid factors of various immunoglobulin isotypes [137]. In the 1950s, Henry Kunkel and his colleagues found proteins in RA with a high sedimentation constant of 22S which proved to be a complex of 19S (RF) and 7S (gammaglobulin) components [138–140]. This led to the novel hypothesis that the RF might be an antibody to antigen-antibody complexes.

The high reported prevalence of RF in the general population ranging from 1.3 to 4% [141] to 21% [142] (and interestingly more than 30% in the North American Pima tribe [143]) challenges the hypothesis that these autoantibodies are pathogenic in principle [144]. Twin studies have shown an association between HLA-DR4 alleles and RF production, and regional differences as documented in Finland suggest environmental factors driving the generation of RF [142]. Bacterial, fungal and most notably viral (hepatitis C) and parasitic infections can lead to the often transient expression of RF suggesting a physiologic role in fighting infections being directed against highly ordered, repetitive antigens as opposed to monomeric or oligomeric antigens [145]. What may be the potential role of infectious agents triggering the production of RF? Most of them indeed display these repetitive epitopes and activate B cells via immune complexes along with Toll-like receptor stimulation with the physiologic goal of immune complex clearance, especially in long standing infections such as Treponema [142].

Although they are found in normal individuals and other diseases, rheumatoid factors are still important humoral features of RA. IgM-RF is easy to detect. Because of its free arms, it has free reaction partners in the test systems. IgG rheumatoid factors stimulate the formation of large immune complexes. In this case, the autoantibodies bind with one another, and it is difficult to separate them from these complexes [146]. Therefore, they cannot be detected in classical rheumatoid factor tests and require other approaches [147]. IgG-RF is a prominent component of the RF response in RA [148]. Additionally, it has been shown that RA synovial fluids from IgM-RF seropositive cases contain high molecular weight IgG complexes as a specific marker of this disease [149]. Somewhat similar complexes may be found in the paired serum of RA patients, but these are considerably smaller, are present at lower concentrations and almost invariably lack the property of activating complement. Analysis of the composition and nature of the complexes revealed that the Fab portion of the IgG component exhibited specificity for determinants on the Fc region of IgG molecules, indicating that the complexes contained IgG-RF. However, the puzzling and unsatisfactory conclusion of these early studies was that at most, 56% of the IgG molecules isolated from IgG complexes could be accounted for as IgG rheumatoid factors, suggesting that still undefined additional antigen-antibody systems are involved in the formation of the synovial fluid complexes.

Nevertheless, in contrast to AMPA, the state of knowledge concerning the RF response in RA has not advanced greatly in recent years and - surprisingly - interest has been low despite the fascinating immunology behind it, since RF represent a prime example of autoimmunity where (auto)antibodies are directed against themselves. As outlined above, RF have been noted to be “an ever-present bystander at the crossroads of host resistance, autoimmunity, and malignancy

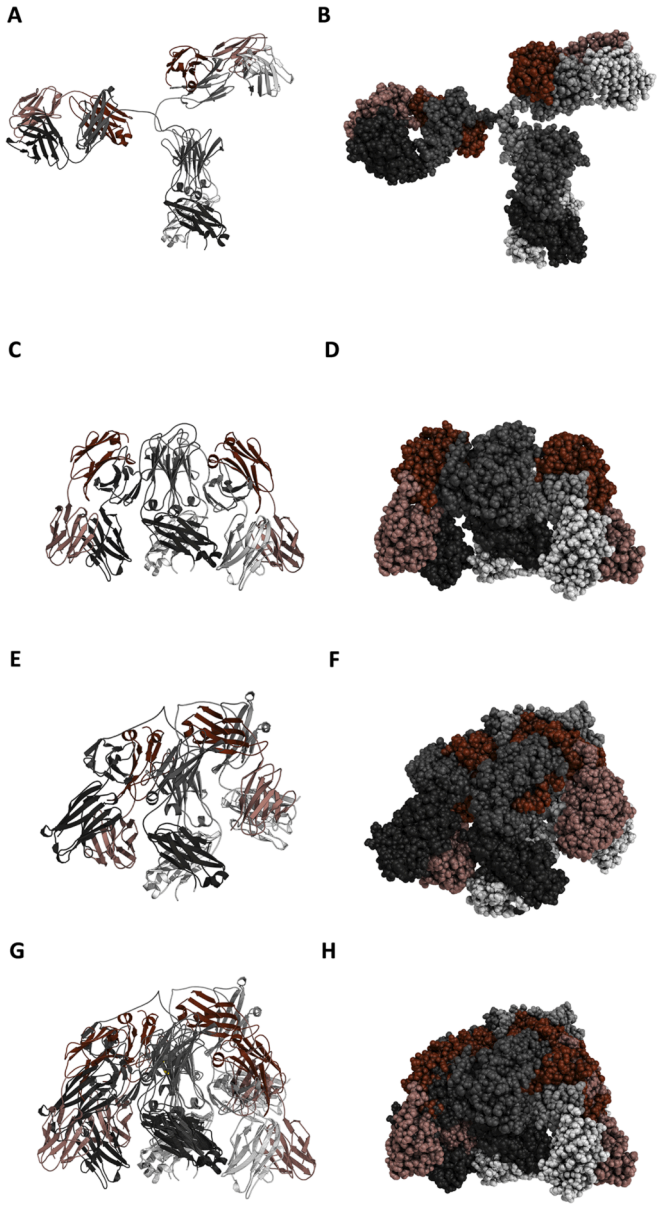

Fig. 3. 3-dimensional modeling of conformational changes of antibody structures that influence the accessibility of the Fc tail for rheumatoid factor binding. Ribbon (left side) and sphere (right side) presentation. A and B: model of an open configuration of an antibody. C and D, E and F: models of two closed configurations that would impede RF-binding. G and H: superimposition of the first and second closed models.
(Maibom-Thomsen et al. (2019), PLoS ONE 14 (6): e0217624, reference 153).

stemming from the viral infection" [144]. What appears to be unique in RA is the fact that still unknown mechanisms cause the abnormal proliferation of RF with class switching to rheumatoid factors of the IgG class, which normally are not detectable. There is abundant evidence that in RA the presence of a positive test for RF implies the presence of distinctive autoantibodies of diverse specificities and isotypes directed to the Fc region of IgG molecules [137,150–152]. However, the nature and origin of the peptide(s) driving the T cell component of the RF response in RA remains unknown and the answer to this puzzling question is likely central to the understanding of this disease. Of particular interest is a recent paper demonstrating that RFs do not bind to native IgG in solution, but only bind to this immunoglobulin once it is complexed with its antigen [153]. Thus, the native state of IgG appears to be present in a closed version, and the Fab arms protect the Fc part and the access to the RF binding epitopes (Fig. 3). This finding may lead to new avenues in studying a special role of IgG once it has bound to its antigen. How this relates to a potential different T cell recognition of IgG derived peptides remains unclear.

Of striking interest, immunization of rabbits with carbamylated vimentin but not with the citrullinated isoform induced a B cell response against the corresponding antigen and against human IgG-Fc. Thus, this experiment provided the first evidence of an interrelation between the anti-Carb immune response and the induction of RF-like reactivity [31]. This finding supports a high level of identity or cross-reaction between peptides that were presented to T helper cells, which subsequently provided help to polyclonal B cells to produce both anti-carbamylated antibodies and RF responses. Based on these results, it will be important to clarify, whether the Fc region of synovial fluid IgG contains citrullinated arginine or carbamylated lysine residues and which peptides are capable to induce an antibody response in RA patients.

6. Synovial inflammation, novel insights

Synovial inflammation in RA is dominated by infiltration of immune cells into synovial tissue (ST) and by joint effusions rich in leukocytes when compared to osteoarthritis. Both, synovial tissue and synovial fluid (SF) have been investigated extensively.

To infiltrate into the joint cavity, cells have to pass the synovial tissue layer, which is very thin in healthy joints but may increase to thick lining layers that mostly consist of macrophages and fibroblastoid synovial cells. Deeper areas of the ST accumulate lymphoid cells, which arrange together with dendritic cells histologically like lymphatic nodules. In parallel, stromal fibroblasts change from a long-stretched phenotype to small round cells, increase by number and present with a proliferative response. These pathologies can be scored [154–157], indicating that the changes are not an on-off phenomenon but express severity and chronicity depending on the magnitude of change. These histological pathologies are also not homogeneous within the same joint but may vary from region to region within the same joint [154]. This heterogeneity of biopsies from the same joint is well known but insufficiently understood, especially when assuming that a putative autoantigen should be distributed evenly and would be expected to induce similar morphological changes all over the joint cavity.

When investigating and counting cells in RA SF, the dominant cell type is the neutrophil granulocyte followed by T-lymphocytes and monocytes/macrophages. Comparing between different arthritic disease entities, cell number and dominance of neutrophils is increasing from almost none in osteoarthritis to the highest concentration and frequency in manifest septic arthritis [158,159]. Although microbes are usually not identified in RA, concentration and frequency of leukocyte subtypes in RA SF is much closer to septic arthritis than to osteoarthritis [160]. Interestingly, the neutrophils, which lead by cell number and frequency in RA synovial fluid, are more or less absent from the synovial tissue, suggesting either different adherence to tissue structures compared to other leukocytes or different qualities of cell type specific attractors. Inversely, cells of the B-cell lineage are almost absent from SF but are clearly represented in the ST compartment. Thus, infiltration of leukocytes seems to be a selective process that results in over-representation of certain immune cell types and not only reflects unspecific extravasation.

From a spatial point of view, destructive processes occur at the invading front of the synovial tissue, the pannus, which predominantly consists of aggressively infiltrating monocytes/macrophages and synovial fibroblasts [161]. These phagocytes have been investigated in the bone marrow, the blood stream and the joint cavity [162]. In RA, there is an increased production of these cells in the bone marrow with premature release and a reduced circulation time in the blood. Cytometric analysis indicates that the monocytes are changing to an activated phenotype, once the cells infiltrate into ST and SF, suggesting either that triggering events occur mostly in the joint or that processes

of extravasation unleash their responsiveness, which may have been primed already in the blood stream. B-cells and also T-cells not only appear deeper in the stromal area but are often located next to blood vessels and are accompanied by dendritic cells. Their histological arrangement comparable to that of lymphatic nodules [163] indicates that processes of antigen presentation by monocytes/macrophages/DC or by B-cells, which requires coordinated and stable cell to cell contact, are easier to reach in the tissue with its anchor surfaces for migration and the presence of chemotactic gradients, which are both absent from the synovial fluid compartment. The chemokine CXCL13 is a typical example for chemotactic processes, as this factor is produced in RA synovium, is the candidate chemokine for B-cell attraction and more or less exclusively associated with appearance of B-cells in inflamed synovial tissues [163]. This factor can be produced by activated monocytes and is a product of activated T-cells, which develop after antigen-dependent successful interaction with APC.

The polarized structure of histological organization raises the question, whether primary triggers in the joint develop in the joint cavity on the surface of the cartilage and induce innate responses of neutrophils and monocytes/macrophages with subsequent involvement of adaptive processes or whether T- and B-cells can induce this aggressive innate response from a distance. So far, the mechanisms, how autoantibodies can explain this type of inflammation in RA are incomplete. Almost all new therapies are directed against cytokines and immune enhancements that emanate from activated monocytes [164] whereas exclusive inhibition of T-cells was of limited success so far [165,166]. In addition, microbiome studies [167] and response to fasting [168,169] suggest that chronic triggers by the gut microbiome may be involved. The possibility of microbial antigen spreading has been discussed for a long time [170] and microbial DNA as well as peptidoglycans have been described earlier as occasional findings in patients with RA [171–173].

Recently Zhang et al. [174] investigated synovial tissue composition in RA and OA using single cell sequencing and characterized 18 different subsets of cells defined on the basis of transcriptional clusters. These consisted of four different subtypes each for synovial fibroblasts, monocytes and B-cells, and three different subtypes each for $CD4^+$ and $CD8^+$ T-cells. Phenotyping by mass cytometry identified some overlap with these transcriptionally defined subsets but also demonstrated that more detailed analysis with both technologies may be necessary to confirm and improve this knowledge. As a first conclusion, some of these cell subsets including $CD90^+$ HLA-DR^{hi} sublining fibroblasts, IL-$1B^+$ pro-inflammatory monocytes, $ITGAX^+$ $TBX21^+$ autoimmune-associated B cells, $PDCD1^+$ peripheral helper T cells and follicular helper T cells and distinct subsets of $GZMK^+$, $GZMB^+$, and $GNLY^+$ $CD8^+$ T cells with particular pro-inflammatory capabilities were proposed as potential key mediators of RA pathogenesis.

Functional interpretation of such data that aim for more detailed resolution on the cellular level is very difficult. As critically discussed before [175] and also indicated by the authors [174], currently achieved resolution by scRNA sequencing provides only limited depth of transcript information when compared to bulk sequencing. Transcripts of many cytokines in the synovial tissue were not detectable by scRNA sequencing but were present in bulk analysis. Furthermore, small populations may arise from cell doublets as cell sorting is not free of errors [176]. Given the high immune cell turnover in inflamed tissues, we have to consider that cells like monocytes may carry transcripts of phagocytosed apoptotic cells, thereby generating new mixed phenotype patterns. Processing of tissue samples by freezing, thawing, enzymatic treatment for cell separation and exclusion of dead cells [177] may generate additional artifacts, which may influence the detection of relative frequencies between the different cell types.

Such technological challenges are reflected by the fact that the 20 best marker genes for each of the 18 clusters (360 genes) were not exclusive to each cell subset, but contained 68 (18.9%) genes belonging to more than one of these transcriptionally defined clusters of cell

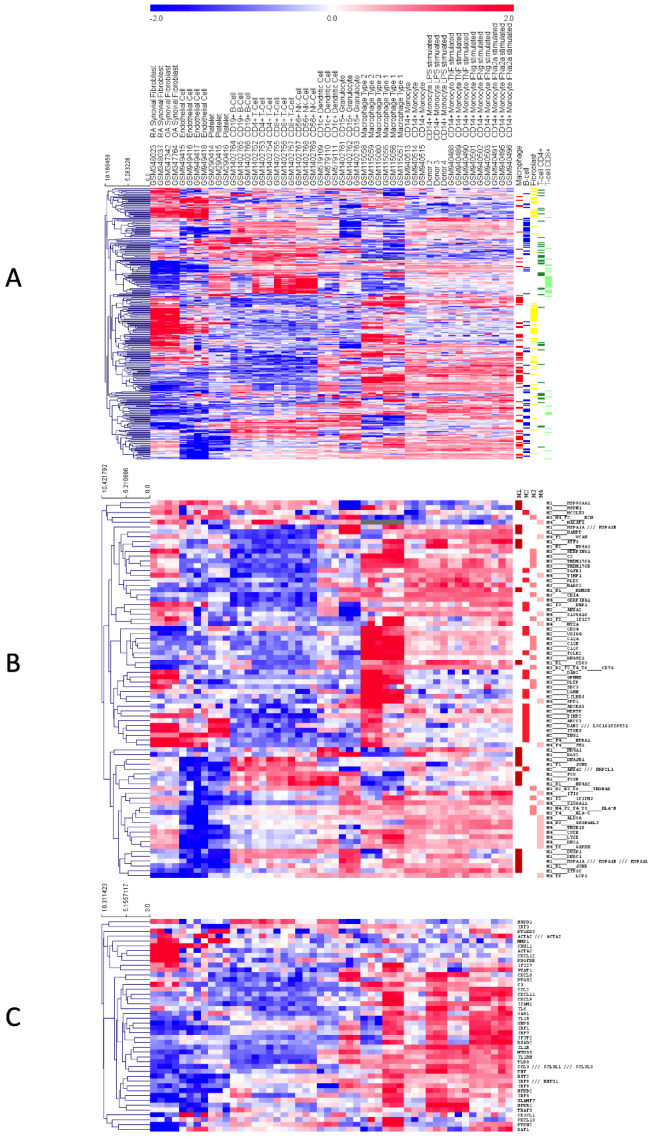

Fig. 4. Matching of synovitis bulk and single cell RNA sequencing genes with cell type transcriptome data determined by microarray hybridization
To characterize genes identified by sequencing of synovitis cells, their expression level was investigated in various cell types profiled by Affymetrix microarray technology. Genes and annotations to cell clusters are derived from sequencing results published by Zhang et al. [174], microarray transcriptome data for clustering in (A), (B) and (C) are derived from the Gene Expression Omnibus (GEO) repository with array-IDs indicated by "GSM …" in the sample name. Signals for clustering were generated by quantile normalization of whole transcriptomes, log2 transformed and z-normalized by gene. (A) presents the hierarchical clustering result with all top 20 genes for the 18 cell type clusters. Genes identified by scRNA sequencing in macrophages (red), B-cells (blue), fibroblasts (yellow), $CD4^+$ (dark green) and $CD8^+$ T-cells (light green) are labelled at the right side of the cluster image. Sequencing annotations to the major cell types are mostly overlapping with microarray based dominant expression in the corresponding cell type. (B) presents the hierarchical clustering result with the top scRNA marker genes associated with macrophage subtypes M1-M4 (dark red to bright red). Only a few genes appear elevated after stimulation of monocytes. (C) presents the hierarchical clustering result with genes identified by bulk sequencing of macrophages, fibroblasts, B-cells and T-cells and consisting predominantly of those genes that belong to significantly enriched pathways of the different cell types. Microarray data indicate that most of these genes are upregulated in monocytes and macrophages when stimulated with LPS, TNF or IFNg and IFNa2a. When compared to (C), only bulk sequencing analysis identified genes related to stimulation processes and revealed several cytokines, which were not identified by single cell analysis.

subsets [174]. Nevertheless, with many of these marker genes, at least the major groups of cell types can be identified when reference transcriptomes of defined immune cells are screened for expression patterns of these genes (Fig. 4A). However, if specific expression patterns of experimentally defined activation states are sought by scRNA sequencing, further differentiation and assignment of individual cell clusters fails due to the limited detection of characteristic markers such as cytokine transcripts (Fig. 4B). In contrast, using the genes identified by sequencing transcripts of whole populations increases the sensitivity for such activation patterns (Fig. 4C). This indicates that an even higher resolution down to individual cells with the currently available technologies does not automatically increase the sensitivity for the detection of the important molecular processes. Successful mapping to microarray-based transcriptome data also illustrates that this pre-existing data can be applied as a valuable resource for the recognition and deconvolution of expression patterns and may even reveal new insights from complex data sets such as whole tissues. Fig. 4C at least demonstrates that bulk sequencing of synovial tissue monocytes identifies activation patterns that overlap with responses to LPS, TNF and IFN stimulation of monocytes. A more detailed analysis of such data by comparison with other stimulation patterns could provide new insight into immune activation processes in RA.

Further support for the involvement of monocytes and macrophages in arthritis was recently presented by Culeman [178] by investigating the spatiotemporal events at the synovial lining in the mouse model. Induction of arthritis triggered local reactions in resident CX3CR1^{+} synovial lining macrophages and changed their organization with disintegration of tight junctions between these cells. These changes may be interpreted as a loss of barrier function, which may drive the progress of inflammation with further influx of immune cells, especially polymorphonuclear cells and monocyte derived macrophages. Processes that would support the local renewing of these resident synovial macrophages could potentially improve the protective barrier function of these cells. This concept of a disturbed monocyte/macrophage homeostasis is also discussed by others [179]. However, if a disturbed homeostasis phenotype is postulated, how can this be maintained in light of constant renewal of monocytes by an increased production in the bone marrow, reduced circulation time in the blood and activation patterns observed only after entering the joint [162]? This constant renewal means that inflammatory monocytes must be replaced. Consequently, it is likely that another mechanism of permanent triggering is still needed.

Investigations of synovial fibroblasts in the animal model by Croft et al. [161] suggested distinct subsets that may drive inflammation and damage. These cells were defined by their expression of fibroblast activation protein-α (FAPα) and differential expression of THY1. Fibroblasts positive for THY1 were identified as immune effector fibroblasts located in the synovial sub-lining whereas those negative for THY1 were characterized as destructive phenotype restricted to the lining layer. Although these cells aggravated inflammation (THY1^{+}) or destruction (THY1^{-}) when injected into inflamed joints, increased numbers of FAPα expressing mesenchymal cells were related to inflammation and declined to baseline levels with resolution of inflammation. When comparing FAPα expression in transcriptomes of complete synovial tissue biopsies from patients [180], RA reveals increased values compared to normal joints, but similar increases are observed in osteoarthritis. THY1 also appears increased in both RA and OA compared to normal synovium but without relevant differences between both diseases. Therefore, confirmatory studies may be necessary, also to clarify the role of these synovial fibroblasts in either driving or responding to inflammation and destruction.

7. Synovial pathotypes and disease associations/outcomes

With the current advancements in synovial tissue analysis, there is growing hope to improve our understanding in pathomechanisms and to identify new targets for treatment. So far, the leading differences relevant for classification and prognosis seem to be more quantitative than qualitative. In order to start with groups suitable for comparative analyses on the basis of new features from high-resolution studies, Zhang et al. separated in leukocyte-rich and leukocyte-poor states [174]. With many new cellular phenotypes suggested by these single cell characterizations, confirmation in larger studies is necessary and already ongoing. Besides this in-depth analysis of synovitis in a cross-sectional study, Lliso-ribera et al. [181] performed a follow-up investigation and included synovial tissue signatures for characterization in the pathobiology of early arthritis cohort (PEAC). This revealed that not only clinical criteria of classification influenced severity but that a lympho-myeloid synovial pathotype requires biologics more frequently to control joint inflammation than pathotypes with diffuse-myeloid or pauci-immune characteristics.

This suggests that different steps of immune cell activation may be dominant in synovitis and that adaptation of immunosuppression strategies to the corresponding steps may at least in part improve the individual outcome. When investigating whole blood transcriptomes in early RA [182], we could find a higher rate of responsiveness to classical DMARDs, if the circulating leukocyte pool was not yet presenting an increase of lymphocyte involvement but was still on a more innate level of activation. Although this was in part influenced by the HLA class II genotype, increased activation of lymphocytes required a therapeutic switch to biologics. Similar observations were reported by Ponchel et al. [183] when analyzing blood leukocytes by cytometry more frequently. This seems to be related to the mode of action when comparing methotrexate (MTX) with anti-TNF therapeutics. The dosing of MTX for RA has its highest impact on the fast renewal of neutrophils [184,185] while blocking of TNF inhibits the switch from the innate defense towards the involvement of adaptive responses via monocytes to T-cells. The more frequent need of treatment with biologics in lympho-myeloid compared to diffuse-myeloid pathotypes confirms this assumption and corresponds with the finding that prognosis is worse in seropositive (lymphocyte activated) compared to seronegative RA, requiring biologics more frequently and earlier.

However, the current efforts to find better molecular targets by studying synovitis with the highest possible resolution must also be critically questioned as to what concrete results can be expected. It will be difficult to further improve the currently limited depth of information from single cell transcriptomes. Although cells and subtypes of cells can be identified, transcripts that belong to characteristic immune stimulations can only be detected using the current sequencing technology in bulk analyses and these stimulation associated genes will presumably lead the way in the identification of new targets. Furthermore, it is to be expected that several biopsies per joint will be necessary. For example, Lliso-ribera et al. [181] have used six biopsies per joint to achieve a reliable assessment of the histological and molecular pathotype.

8. Conclusion

The causes of RA are still unclear, even though important progress has been made in recent years. In particular, our understanding of the immunological changes in the pre-disease phase and in the earliest phases of arthritis has improved considerably, as has the granularity to which we can now disect the cellular composition of the tissues at the site of inflammation. The current strategy of immunosuppression based on autoimmune inflammation has essentially improved the symptoms and delayed destructive progress, but not cured the disease. In view of the increasing research results from studies of the microbiome and the rapid improvement of the symptoms under fasting [168,169], recurrent or permanent microbial triggers are not unlikely to play an important role in the disease. This etiological link would be compatible with the substantial innate immune activation of granulocytes and monocytes especially in the joint and the increase and changes of adaptive

immunity over time. It will be intriguing to see how continuous research in the new decennium will evolve and shed light on how these aspects relate.

We are delighted to also meet the wish of the editor to add a personal note for Josef Smolen, who has been an ardent student of rheumatoid arthritis. He started very early in his career with investigating the role of antibodies in arthritis models using collagen-anticollagen immune complexes as a tool to study the fascinating pathogenic role of immune complexes in general [186]. He also demonstrated anti-collagen antibodies in the synovial fluid of RA patients using the then innovative technology of radioimmunoassays [187]. Moreover, he tested the hypothesis of the role of autoantibodies by employing plasmapheresis [188]. Along with Eng Tan he addressed the etiology of RA stressing the importance of rheumatoid factors in an excellent review [189]. But of course, studying the etiopathogenesis and the clinical features of RA is not enough. Ultimately, novel therapeutic avenues are needed to battle this disease that uncontrolled has a devastating course. Josef Smolen has been a leading and outstanding researcher, clinician scientist and physician to fight destruction, pain, loss of function and social deprivation caused by this disease. In addition to his involvement in the new ACR/EULAR classification of RA and the definition of remission, we believe that two achievements are of prime importance: first the targeted therapy strategy [190] and second the EULAR management recommendations [191]. These endeavors have indeed paved the way to a new life for our patients and certainly continue to do so.

Acknowledgements

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