

The pathogenic potential of autoreactive antibodies in rheumatoid arthritis

Marieke Bax · Tom W. J. Huizinga · René E. M. Toes

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Abstract Rheumatoid arthritis (RA) is an autoimmune disease affecting ~1 % of the population. Although major advances have been made in the treatment of RA, relatively little is known about disease pathogenesis. Autoantibodies, present in approximately 60 % of the patients with early disease, might provide indications for immunological mechanisms underlying RA. Among the RA-associated autoantibodies, especially anti-citrullinated protein antibodies (ACPAs) have been studied intensively in the last decade. The discovery of ACPAs resulted into novel insight in RA pathogenesis and allowed division of the heterogeneous entity of RA into an ACPA-positive and ACPA-negative subset of disease. Other autoantibodies discovered in the serum of RA patients, including rheumatoid factors (RFs) targeting human IgG and anti-peptidylarginine deiminase (PAD)3/4 antibodies reactive against and activating the enzyme involved in citrullination, might contribute in collaboration with ACPAs to a feed-forward loop to aggravate erosive outcome of disease. Recently, a novel autoantibody system associated with RA was identified. These autoantibodies recognize carbamylated proteins (anti-CarP antibodies) and are detected in approximately 20 % of ACPA-negative patients, suggesting another parameter to sub-classify RA. In this review, the implication of autoantibodies in RA pathogenesis, diagnosis, prognosis and as biomarker for personalized medicine is discussed.

Keywords Rheumatoid arthritis · Autoantibodies · ACPAs · Anti-CarP antibodies · Posttranslational modifications · Autoreactive B cells

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M. Bax · T. W. J. Huizinga · R. E. M. Toes (✉)
Department of Rheumatology, Leiden University Medical Center,
PO Box 9600, 2300, RC Leiden, The Netherlands
e-mail: R.E.M.Toes@lumc.nl

Introduction

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting 0.5–1 % of the population in industrialized countries. RA is characterized by persistent synovitis, systemic inflammation and the presence of several autoantibodies. The clinical course is extremely variable, showing a wide spectrum of clinical manifestations ranging from mild and self-limiting disease to rapidly progressive inflammation with joint destruction and severe physical disability [1, 2]. Because of the heterogeneous character of the disease, classification criteria have been developed to provide a basis for disease definition and to standardize recruitment in clinical trials and comparison of the results of multi-centred studies. Since 1987, RA has been classified based on the ACR 1987 criteria defined by a regression analysis of disease characteristics of “classic cases”. This resulted in the inclusion of a heterogeneous set of patients with conceivably different “pathogenic” backgrounds [3]. The ACR 1987 criteria are well accepted for disease definition, but have a significant limitation in identification of patients with recent-onset RA. Especially, this latter subset of patients would benefit from early effective intervention to avoid progress to the chronic, erosive state of RA exemplified in the 1987 criteria. Therefore, ACR/EULAR 2010 criteria were developed [4], which display a higher sensitivity but a lower specificity for RA to be able to diagnose RA and start treatment early in disease.

The heterogeneous character of RA is also reflected by a wide range of responses to therapies. Patients do not respond uniformly to therapeutics, conceivably because the specific biological pathway targeted by the drug may not be active in the particular patient subgroup. Currently, it is difficult to predict effectiveness of a specific therapy in individual patients because we lack knowledge to sub-classify RA based on

biological pathways involved in pathogenesis. For the development of a personalized medicine approach or even more ambitious, curative therapeutics, it is essential to understand the immunological mechanisms underlying RA. Although RA is a considerable health problem, still relatively little is known on its immunopathology. Refinement of the understanding of molecular pathways involved in disease pathogenesis could be achieved by combined knowledge on RA-associated genes, environmental factors and the presence of serological factors.

Genetic and environmental contributions to RA

Both genetic and environmental risk factors contribute the development of RA. Smoking is the most prominent environmental factor, with its largest effect in autoantibody-positive patients [5, 6]. Although the prevalence of RA in the general population is <1 %, among monozygotic twins with one of them affected, for the unaffected sibling, the prevalence increases to 12.3 to 15.4 %. This implicates that genetic factors have a substantial impact on susceptibility to RA, with the genetic contribution estimated around 50 to 60 % [7]. The identification of disease-associated genes provided valuable insight into biological pathways that might contribute to RA pathogenesis. The strongest predisposing variants of the genetic risk factors are found in the human leukocyte antigen (HLA) alleles, accounting for 30 to 50 % of overall genetic susceptibility to RA [8, 9]. Already in 1969, it was demonstrated that in mixed lymphocyte cultures, peripheral blood lymphocytes from RA patients were non-reactive to each other [10] and that this non-reactivity was due to the sharing of genes in the HLA region [11]. Subsequently, it was discovered that multiple RA risk alleles within the HLA-DRB1 gene share a conserved amino acid sequence, leading to the “shared epitope” (SE) hypothesis [12]. The odds ratio to develop RA for one HLA SE allele is around 4, whereas two SE copies increase the odds ratio to approximately 12 [13]. The underlying mechanism by which HLA SE alleles predispose to the development of RA is still not understood, despite much progress in perception of the structure and function of HLA-DRB1 molecules. HLA-DR molecules are anchored in the membrane of antigen-presenting cells to present antigenic peptides to T lymphocytes. The T cell receptor recognizes residues from both the peptide as well as the HLA-DR molecule itself. The SE motif is situated at the part of the HLA-DR molecule that binds to the peptide. At this position, the SE influences peptide binding and presentation to T cells (Fig. 1). So far, no specific arthritogenic peptides have been identified that bind to SE HLA-DR molecules and contribute to RA.

It was not until 2001, almost 30 years after the identification of HLA-alleles as a risk factor for RA, that non-HLA loci associated with RA were revealed [14]. Interestingly, one of these loci encode the gene for PADI4, an enzyme that citrullinates proteins and hereby creating targets for anti-

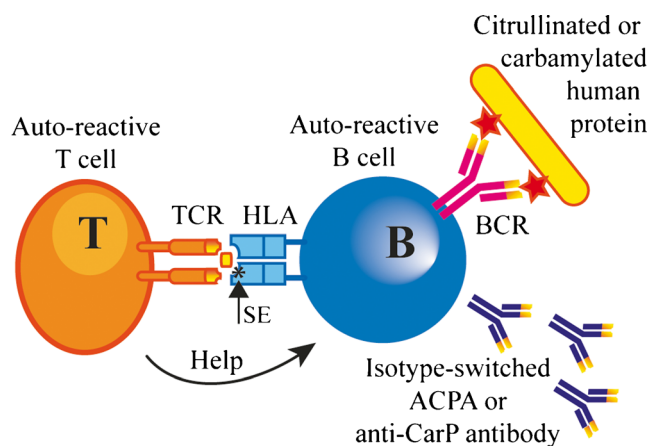


Fig. 1 Antigen-activated B cells require help from antigen-specific T cells to secrete isotype-switched antibodies. B cells recognizing citrullinated or carbamylated human proteins internalize antigen with their B cell receptor. After processing, antigen is presented to T cells on HLA-DR molecules anchored in the B cell membrane. The SE motif is situated at the part of the HLA-DR molecule that binds to the peptide and influences at this position peptide binding and subsequent presentation to T cells

citrullinated protein antibodies (ACPAs) [15]. This directly implicates citrullination as important mechanism for RA development. After 2004, the identification of new genetic risk factors underwent an unprecedented acceleration. Genome-wide association studies rapidly expanded the number of genes associated with ACPA-positive RA. Currently, more than 30 genetic risk factors have been identified [16]. Most of them are located near genes that are linked to T or B cell activation or differentiation, and cytokine signalling, implicating—not surprisingly—immune-related events as an important component of RA-disease pathogenesis [17].

In addition to genes predisposing for RA, also, protective genes that are more often present in healthy controls compared to RA patients have been identified. A meta-analysis was performed to investigate which HLA-DRB1 alleles are associated with protection in ACPA-positive RA and ACPA-negative RA, stratified for SE alleles. In ACPA-positive RA, the alleles that conveyed protection after stratification for SE were HLA-DRB1*13 alleles, containing the amino acid DERAA. In ACPA-negative RA, no robust associations were found with protective HLA-DRB1 alleles [18].

RA-associated autoantibodies

The presence of autoantibodies in serum of RA patients has been recognized already for over 70 years. Rheumatoid factor (RFs), targeting the Fc part of human IgG were the first group of autoantibodies discovered [19]. Unfortunately, RFs do not have high specificity for RA and can be detected in up to 15 % of the healthy individuals. In 1964 and 1979, additional serum factors present in RA patients were described, anti-perinuclear factor antibodies and anti-keratin antibodies, respectively [20,

and B cell receptor triggering followed by (autophagy-facilitated) HLA-restricted citrullinated antigen presentation to T cells, ultimately resulting in breach of T cell tolerance and development of ACPA responses (Fig. 1). To dissect the events that occur in the break of tolerance against citrullinated proteins, animal models of arthritis could be used. However, it is unclear at the moment whether a genuine ACPA response can be induced in animal models of arthritis [30, 31].

Carbamylated proteins contain a homocitrulline residue that has been generated through a chemical reaction in which mainly lysine is converted into a homocitrulline through the action of cyanate. Cyanate is naturally present in the body and in equilibrium with urea [32]. Under steady-state physiological conditions, the cyanate concentration might be too low to allow extensive carbamylation. Indeed, in renal failure, the urea concentration increases, resulting in extensive carbamylation of proteins [33]. Interestingly, smoking, the most prominent environmental risk factor for RA, increases the cyanate concentration and might consequently enhance carbamylation. However, most carbamylation is believed to be provoked under inflammatory conditions, when MPO is released from neutrophils. MPO converts thiocyanate to cyanate, allowing extensive carbamylation [34]. RA is characterized by inflamed joints; therefore, it is conceivable that carbamylation is a naturally occurring process in the inflamed synovium.

In addition to posttranslational modifications themselves, also, autoantibodies against the enzymes involved in generating posttranslational modifications are described in RA [35–38]. It was recently discovered that a subset of anti-PAD4 autoantibodies increase the catalytic efficiency of PAD4 by decreasing the enzyme's requirement for calcium [25]. This implies that anti-PAD autoantibodies might be involved in the generation of the ligands for ACPA.

Characteristics of RA-associated autoantibodies

ACPA antigen recognition

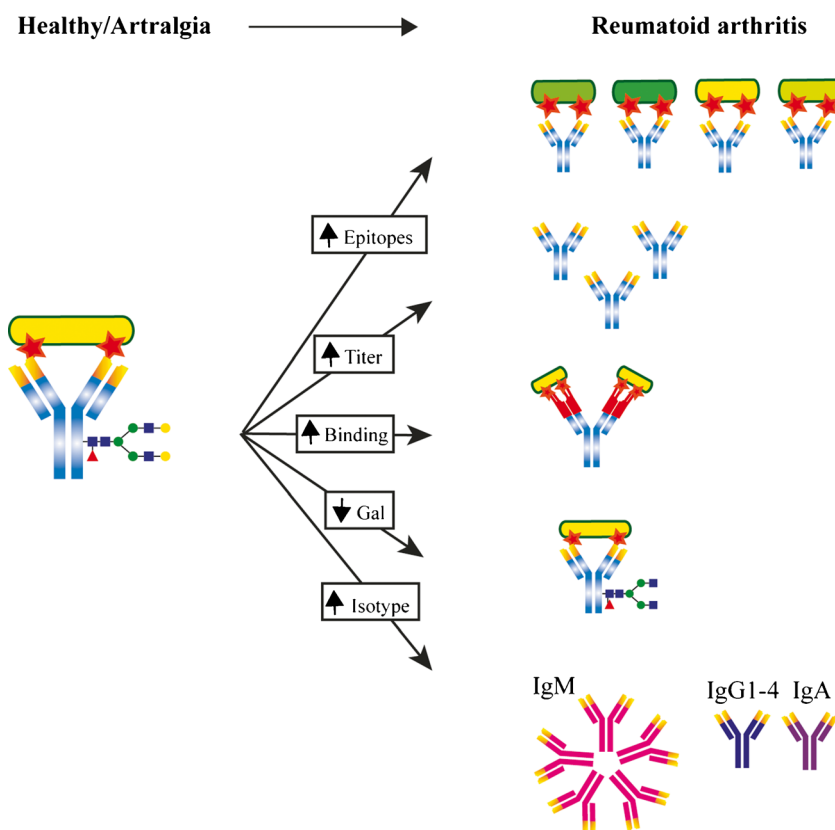
ACPAs recognize posttranslationally modified proteins with the “non-encoded”, amino acid citrulline generated after enzymatic conversion of arginine. The first enzyme-linked immunosorbent assay (ELISA) to detect ACPA based upon the molecular identity of the antigens recognized by ACPA was developed in 1998, using citrullinated peptides derived from several filaggrin epitopes [39]. To allow routine testing, in 2002, the first commercial ELISA test was available with CCP to detect ACPA as biomarker in RA. This anti-CCP ELISA proved to be extremely specific (98 %) for RA and had a significantly higher specificity in comparison with the IgM RF ELISA [40]. To confirm that the antibodies detected by CCP ELISA are in fact ACPA, it was shown that affinity-purified

anti-CCP antibodies were able to recognize citrullinated proteins on Western blot. This indicates that anti-CCP antibodies recognize multiple citrullinated proteins and are a collection of ACPA. To determine to what extent antibody responses to different citrullinated antigens are cross-reactive, a method was established in which citrullinated antigen-specific antibodies were eluted from ELISA plates and then used for detection of other citrullinated antigens with Western blot or in ELISA, with citrullinated or control peptides as inhibitor. These experiments showed that antibodies specific for citrullinated fibrinogen and citrullinated myelin basic protein were cross-reactive. This cross-reactivity is not complete, as distinct non-cross-reactive responses can also be detected in RA patients [41]. Correspondingly, murine monoclonal ACPA cross-reacted with variable degrees to citrullinated epitopes on different peptide backbones [42]. Furthermore, by single B cell-based cloning technology it was demonstrated that human citrulline-reactive monoclonal antibodies reacted with more than one citrullinated antigen [43], confirming the cross-reactive nature of ACPA. This cross-reactivity towards different citrullinated proteins indicates that citrulline is recognized in a hapten-like manner to elicit immune responses towards several (self-) proteins.

Epitope spreading, an increase or shift in the antigen recognition profile, can have important pathophysiological consequences in autoimmune diseases. It was demonstrated that ACPA epitope spreading occurs several years prior to the onset of clinical RA [44]. At later stages of the disease course, the ACPA fine specificity does not change anymore [45]. Therefore, it is suggested that the initial autoimmune response is directed towards a limited, but not always the same set of autoantigens. At a later stage in the development of the autoimmune response, an increasing number of epitopes are recognized. This is paralleled by an increase in ACPA levels which also occurs before onset of symptoms; after which, both the ACPA-epitope recognition profile and the level of ACPA stabilize to relatively high levels at onset of disease [44] (Fig. 3). It is estimated that in ACPA-positive RA patients, up to 1 in 80 IgG molecules in serum are ACPA, indicating that absolute ACPA levels are high compared to other antibodies in established disease [46].

Within the ACPA repertoire, the presence and detection of different fine specificities offered hope for the possibility to use the ACPA epitope recognition profile as biomarker to predict disease development and progression. Although epitope spreading occurs on the group level before patients fulfill the ACR 1987 criteria for RA, current evidence indicated that this does not correlate with clinical outcome as measured by rate of joint destruction. For example, no statistically significant differences in clinical or in radiological outcomes were demonstrated between the ACPA recognizing citrullinated enolase peptide 1 (CEP1)-positive and negative subset of RA patients [47]. Since ACPA-positive patients display a heterogeneous ACPA recognition profile, cluster analyses have been

Fig. 3 ACPA maturation in RA development. Before onset of arthritis, the epitope recognition profile, antibody levels, avidity for its antigen, glycosylation pattern and isotype usage of ACPA change



performed in an attempt to identify subgroups of patients on the basis of their ACPA recognition profile. No apparent clustering of patients was found, and the recognition of specific citrullinated epitopes was not associated with baseline characteristics. Furthermore, patients with an extended fine-specificity repertoire did not display differences in baseline characteristics or joint damage after 7 years of follow-up compared to ACPA-positive patients recognizing few peptides [48], also indicating the limited use to detect ACPA fine specificities for diagnostic or prognostic purposes.

ACPA and the HLA system

Intriguingly, the strong association between SE-encoding HLA-DRB1 alleles and RA is only observed for ACPA-positive disease [13]. It was demonstrated that the HLA-SE-alleles influence not only the magnitude but also the specificity of the ACPA response by the finding that HLA-SE alleles predispose to the development of antibodies against citrullinated vimentin peptides, but not to the development of antibodies against citrullinated fibrinogen peptides [49]. However, although several ACPA fine specificities are more readily formed under the influence of SE alleles, again, no association between the presence of the HLA-SE alleles and radiological damage was observed in the ACPA-positive patient group [50]. These data indicate that although the HLA-SE alleles are instrumental in shaping the ACPA-repertoire,

the “shape” of the ACPA-repertoire does not translate to meaningful clinical differences.

ACPA isotype switching

Antigen-activated B cells initially secrete antibodies of the IgM isotype, and after activation by T helper cells, they undergo isotype switching to produce IgG, IgA or IgE antibodies (Fig. 1). Isotype switching is a process in which the constant region of the antibody is changed, to affect the effector function of an antibody, whereas the variable region of the antibody, important for antigen recognition, is not affected. ACPA have been found in different forms, including IgG, IgA and IgM. The fine specificity and isotype usage of ACPA in health and disease differs. The ACPA-response present in healthy family members of patients with RA uses fewer ACPA isotypes than ACPA from RA patients [51]. Nonetheless, ACPA of both the IgG and IgA isotypes predate the onset of RA by years and predict the development of RA with the highest predictive value for IgG antibodies [52]. Although the usage of isotypes by ACPA increases during the development towards RA [45], the ACPA isotype distribution does not expand in established disease [53], indicating that most of the expansion of isotype usage by ACPA takes places before the onset of arthritis (Fig. 3). The number of isotypes used by ACPA does not only associate with RA development but also with RA progression, as the magnitude of the ACPA

isotype profile at baseline reflects the risk of future radiographic damage [54].

ACPA avidity maturation

To evaluate if the evolution of the ACPA response differs intrinsically from the protective responses against pathogens, the avidity of ACPA in relation to the avidity of antibodies against recall antigens has been analysed. Intriguingly, the avidity of ACPA was significantly lower than the avidity of antibodies to the recall antigens tetanus toxoid and diphtheria toxoid. Moreover, ACPA did not show avidity maturation during longitudinal follow-up, even in patients who displayed extensive isotype switching [55]. These observations indicate that the evolution of ACPA differs from the development of antibodies against recall antigens as isotype-switching and avidity maturation are apparently uncoupled. The reason for low avidity of the ACPA response compared to “conventional” antibody responses against recall antigens in the same patients is unknown. However, it is conceivable that these observations are related to the notion that citrullinated antigens are abundantly expressed in the human body, including B cell follicles in lymph nodes. This avoids competition for only the strongest antigen-binding B cell receptors to bind these citrullinated antigens to receive survival signals. Abundant expression of citrullinated antigens could also explain the presence of high levels of IgM ACPA in RA patients [46]. Often, the presence of IgG prevents priming of new IgM-positive B cells, as a consequence of higher avidity for the same antigen. This principle is, for example, used in the context of Rhesus-antigen immunity, where a mother of a Rhesus-positive child is injected with anti-Rhesus IgG shortly after delivery, to prevent the formation of a new anti-Rhesus response by low-binding anti-Rhesus IgM from the mother. Probably, IgG ACPA is not able to capture the citrullinated antigens preventing them from binding to IgM-producing B cells, because of the low avidity of the IgG ACPA and/or the high concentration of citrullinated antigens. Interestingly, the detection of IgM ACPA also indicates that the presence of a continuous ongoing ACPA-immune response as IgM has a short half-life and is highly expressed in the synovial compartment [46].

Glycosylation of ACPA

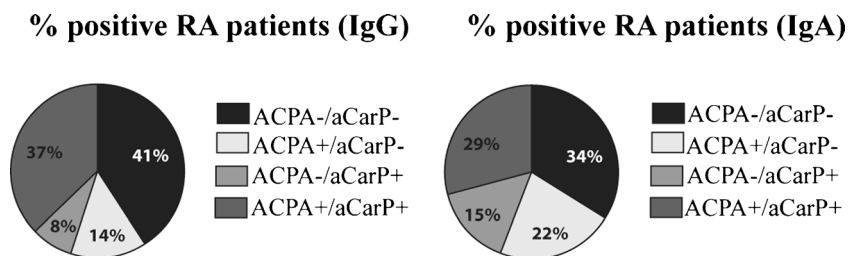
Already in 1985, the importance of antibody glycosylation in RA was acknowledged. To obtain more information on autoantigenic reactivity of RF, Parekh et al. compared in detail the N-glycosylation pattern of serum IgG isolated from normal individuals and from patients with RA. The results of this study indicated that disease is associated with changes in the relative extent of galactosylation of IgG antibodies. The investigators proposed that RA might be a “glycosylation

disease”, reflecting changes in the intracellular processing or postsecretory degradation of N-linked oligosaccharides [56]. After the discovery of ACPA, IgG ACPA glycosylation was determined, to address the occurrence of specific glycosylation features of antigen-specific subpopulations of antibodies. Glycosylation patterns of ACPA-Fc were analysed because glycosylation of the Fc part can underlie pro- versus anti-inflammatory characteristics of antibodies [57]. It was demonstrated that Fc glycosylation of IgG1 ACPA varies considerably from total serum IgG1 of patients with respect to galactosylation patterns [58]. Since RA is characterized by inflammation of the synovium, Fc glycosylation of IgG1 ACPA derived from serum and synovial fluid was compared. It was demonstrated that Fc glycosylation of ACPA differs considerably between synovial fluid and serum [59]. This might indicate that an inflammatory environment contributes to antibody glycosylation profiles. Indeed, environmental factors, including retinoic acid, TLR ligands and cytokines have specific impact on IgG1 Fc-glycosylation patterns, whereas no altered glycosylation patterns were observed for total cellular glycan proteins [60]. Because ACPA is present years before onset of disease and some molecular characteristics of ACPA change before disease onset, it was investigated whether ACPA exhibit specific changes in Fc glycosylation prior to the onset of arthritis. ACPA-IgG1 from arthralgia patients display Fc glycosylation patterns comparable to that of asymptomatic blood donors. However, around 3 months before disease onset, ACPA display significant changes in Fc galactosylation and fucosylation [61], again, indicating that specific changes in the molecular composition of ACPA occur before clinical precipitation of disease (Fig. 3).

Anti-CarP antibodies

The chemical structure of homocitrulline highly resembles citrulline (Fig. 2). Therefore, it was hypothesized that ACPA might recognize homocitrulline present on proteins (carbamyated proteins) as well. Surprisingly, it was shown that most ACPA did not recognize peptides in which the citrulline residue was replaced by a homocitrulline residue, indicating that ACPA can discriminate between citrulline and homocitrulline. Intriguingly, reactivity towards homocitrulline containing antigens was noted in some patients in these studies. Therefore, an assay was developed to detect antibodies against carbamyated proteins. Interestingly, it was demonstrated that 45 % of the RA patients harbour IgG antibodies recognizing carbamyated proteins. Of note, not only ACPA-positive RA patients showed anti-CarP reactivity but also 16–30 % of the ACPA-negative patients harboured anti-CarP antibodies (Fig. 4) [24]. This observation demonstrated the identification of a novel group of autoantibodies present in RA. Since the discovery of this unique novel autoantibody system in RA was only a few years ago, the molecular

Fig. 4 Percentage of RA patients positive for IgG ACPA and anti-CarP antibodies and IgA ACPA and anti-CarP antibodies



characteristics of anti-CarP antibodies are to be defined. Likewise, it is currently not known whether carbamylated proteins are present in (inflamed) joints. The possibility to induce anti-CarP immunity by vaccination with carbamylated and citrullinated proteins [62, 63] and the emerge of anti-CarP antibodies upon arthritis induction in animal models (Stoop et al., submitted for publication) might allow to study the driving mechanisms underlying the anti-CarP immune response and their contribution to arthritis.

Pathogenic potential of RA-associated autoantibodies

The strong association with RA suggests a prominent role for ACPA in disease pathogenesis. The presence of ACPA in patients already diagnosed with RA predicts severe joint damage [64], whereas the presence of ACPA predicts progression towards RA in patients with undifferentiated arthritis [65]. Furthermore, the presence and levels of ACPA are indicative for arthritis development in arthralgia patients [66, 67]. ACPA-positive arthralgia patients show local subclinical inflammation in small joints already in the pre-clinical phase [68]. Likewise, it was demonstrated that most ACPA-positive RA patients appear to be ACPA positive already years before onset of disease [69]. In addition, polymorphisms in genes encoding the PAD enzymes associate with RA [15], indicating that the enzymes generating antigens for ACPA are involved in pathogenesis. Nonetheless, although suggestive, these observations do not show direct evidence of ACPA implication in disease pathogenesis.

Effector functions of ACPA

Intriguingly, shortly before the start of clinical symptoms, the epitope recognition profile, isotype usage, avidity maturation and glycosylation pattern of ACPA change [44, 45, 52, 61, 70] (Fig. 3), indicating that maturation of the ACPA response is required for its pathogenicity. Likewise, the magnitude of the ACPA isotype profile at baseline is associated with radiographic damage, showing an odds ratio of 1.4-fold increase for every additional isotype [54]. The mechanism behind elevated disease severity in association with isotype usage is still unknown; however, isotype usage might be implicated in the effector functions recruited by ACPA. These include

complement activation [71], activation of Fc-receptor positive cells [72] and osteoclast activation. The presence of ACPA was associated with serum markers for osteoclast-mediated bone degradation. Interestingly, ACPA bind to osteoclast surfaces, resulting in osteoclastogenesis and bone degradation. This was confirmed by adoptive transfer of human ACPA into mice, leading to osteopenia and increased osteoclastogenesis [73].

Indications for a pathogenic role of ACPA were found in the synovium of ACPA-positive RA patients as well. Histological differences, mainly with regard to infiltrating lymphocytes, in the inflamed joint of ACPA-positive and ACPA-negative RA patients have been visualized [74]. Indeed, targets for ACPA are present in joints of RA patients, demonstrated by the detection of many citrullinated proteins in synovial fluid samples from the inflamed joints of RA patients [75]. Because ACPA display lower avidity to its antigen compared to antibodies against recall antigens [55], ACPA avidity in relation to biological activity and clinical outcome was analysed. Unexpectedly, patients with low-avidity ACPA display a higher rate of joint destruction [76]. The mechanisms behind this remain elusive, but could be due to a higher ability of low-avidity antibodies to penetrate tissues.

ACPA and neutrophil extracellular traps

The strong connection of HLA with (ACPA-positive) disease [13] elicits the focus of RA research on the adaptive immune system. Recently, a connection between the innate and adaptive immune system in RA was proposed by the observation that PAD4 is required for neutrophil extracellular trap (NET) formation by neutrophils [77]. NETs are highly decondensed chromatin structures that are formed by neutrophils to trap and kill bacteria. The observation that PAD4 is required for NETosis is interesting, because enhanced NET formation by neutrophils has already been shown to be related to autoimmunity, potentially because autoantigens together with immunostimulatory molecules are exposed to trigger the immune system. NETosis was directly linked to RA by the demonstration that citrullinated histone 4 from NETs is a target for RA sera [78]. Furthermore, enhanced NETosis in neutrophils derived from peripheral blood and synovial fluid of RA patients correlates with ACPA levels. Interestingly, immunoglobulin fractions from RA patients with high levels

of ACPA and/or RF are able to significantly enhance NETosis in neutrophils from RA patients and these NETs induced by antibodies from RA serum harbour citrullinated proteins. Intriguingly, also, purified human ACPAs have been reported to induce potent NETosis in RA neutrophils [79]. This indicates that ACPA can enhance the formation of NETs by neutrophils, resulting in expel of immunostimulatory molecules together with its own target, citrullinated autoantigens. These observations suggest a mechanism that may promote and perpetuate disease (Fig. 5).

Pathogenic potential of anti-PAD3/4 antibodies

Similar to ACPA and anti-CarP antibodies, anti-PAD4 antibodies have been associated with severe disease in established RA [35, 36]. Like RF, anti-PAD antibodies are associated with the presence of ACPA. Although anti-PAD4 antibodies have been detected with a mean duration of 4.7 years prior to the clinical diagnosis of RA in a small subset of patients, in the majority of patients, anti-PAD4 antibodies were detected after the appearance of ACPA [80]. To assess anti-PAD antibodies in health and disease, the presence of anti-PAD4 antibodies was tested in first-degree relatives of indigenous North American people with RA who are characterized by a high prevalence of ACPA. Interestingly, in contrast to ACPA, anti-PAD4 antibodies were almost exclusively found in people with established RA [81].

Recently, it was discovered that a subset of anti-PAD4 antibodies increases the catalytic capacity of PAD4 by decreasing the calcium requirement of this citrullination enzyme. The identification of autoantibodies that activate an enzyme that itself generates antigens for another group of autoantibodies associated with RA identifies an important feed-forward loop. This might drive worse outcome of disease and therefore potentially identify patient subsets that require aggressive treatment. The predictive value of anti-PAD

antibodies for severe disease was confirmed by the finding that PAD3/PAD4 cross-reactive autoantibody-positive RA patients showed increased radiographic damage compared to PAD3/PAD4 cross-reactive autoantibody-negative individuals [25].

Autoreactive B cells

The introduction of biologicals for RA treatment provided valuable insight into the contribution of specific immunological mediators in pathogenesis. The effectiveness of biologicals depleting B cells, such as rituximab, demonstrates evidence for the involvement of B cells and/or (auto) antibodies in disease pathogenesis. In conventional B cell activation, which is intensively studied for vaccination strategies, antigen binding to the B cell receptor initiates signalling pathways for somatic hypermutation. After somatic hypermutation, B cells with the highest affinity receptors for their antigen are selected for survival. This process continues during evolution of the immune response, resulting in competitive survival and proliferation of B cells with high-affinity receptors. In contrast, low-affinity B cells die by apoptosis and eventually disappear from the population. Interestingly, ACPA-producing B cells behave differently, demonstrated by the identification of the avidity of ACPA for its antigen significantly lower than the avidity of antibodies to the recall antigens tetanus toxoid and diphtheria toxoid [55]. The avidity of ACPA is even low after extensive isotype switching [51], indicating that in ACPA-specific B cells, affinity maturation and isotype switching, in contrast to general B cell biology, is uncoupled.

Recently, a culture method has been developed to detect the presence of ACPA-producing B cells in different compartments of the human body and to study the characteristics and phenotype of these cells derived from RA patients. B cells were isolated from peripheral blood or synovial fluid from ACPA-positive RA patients and cultured with or without stimulating factors to assess the presence of ACPA-producing B cells in the different compartments. It was estimated that around 1 in 20,000 B cells in blood of RA patients secrete ACPA [82] versus around 10–20 % of the B cell population present in the synovial fluid compartment (Kerkman et al., unpublished observations). Likewise, it was demonstrated that ~25 % of the synovial IgG-expressing B cells from ACPA-positive RA patients recognize citrullinated autoantigens [43]. Furthermore, it was shown that IgM-ACPA is the most abundantly present isotype of ACPA in synovial fluid, with the highest enrichment in the range of one IgM-ACPA for every eight IgM-antibodies [46]. Analysis of the BCR repertoire in blood and synovium showed multiple dominant clones in inflamed synovium and hardly any in blood. Interestingly, the fraction of IgM clones was higher in established RA compared to early RA [83]. Together, this

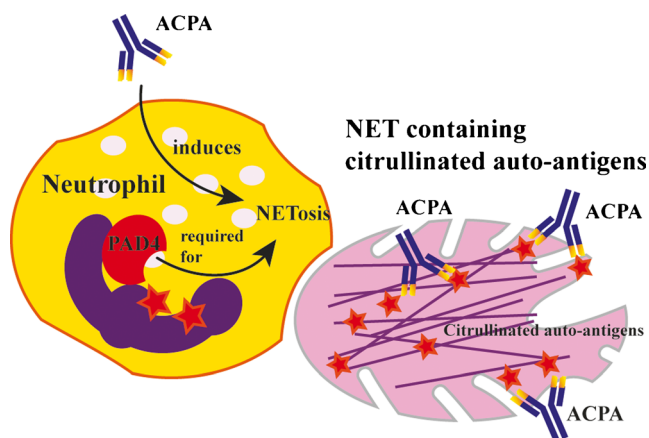


Fig. 5 ACPA have been reported to enhance the formation of NETs by neutrophils, resulting in expel of immunostimulatory molecules together with citrullinated autoantigens, the targets of ACPA

indicates that the synovium is a niche for expanded autoantibody-producing B cells and is the décor of an ongoing induction of anti-citrulline immune responses in RA patients.

Autoantibodies as biomarkers for early disease and disease sub-classification

To prevent development towards the classical picture of RA, with massive erosions and deformities of inflamed joints, early treatment intervention is very important. Currently, it is hard to predict whether arthralgia and undifferentiated arthritis (UA) patients will go in remission or will develop RA. To decide which patients would benefit from aggressive intervention, robust biomarkers are required to diagnose RA early in disease.

RA is characterized by a wide spectrum of clinical manifestations, ranging from mild and self-limiting disease to rapidly progressive inflammation, joint destruction and severe physical disability. This heterogeneous character of RA is reflected by a broad range of responses to therapies. Currently, we are unable to predict the effectiveness of a specific therapy in individual patients, because we lack robust biomarkers for RA sub-classification. The discovery of the serological marker ACPA was of outstanding impact because for the first time, it was possible to sub-classify the heterogeneous entity of RA based on serological markers. ACPA-positive and ACPA-negative RA patients display major differences regarding genetic and environmental determinants of disease, molecular features of the affected joint, remission rates and, importantly, response to treatment.

Already before RA establishment, it could be considered to treat ACPA-positive UA patients differently than ACPA-negative UA patients. It was demonstrated that in ACPA-positive UA patients treated with methotrexat, RA progression was circumvented or delayed. In contrast, for the ACPA-negative subset of UA patients, no effect of methotrexat was observed [84]. Furthermore, ACPA-positive UA methotrexat responders had lower levels of IgG ACPA before start of the treatment compared to ACPA-positive UA non-responders [85]. This suggests not only that different treatment strategies for ACPA-positive versus ACPA-negative patients might be required but also that in UA patients with high ACPA levels, only methotrexat treatment might not be sufficient. In established RA, different treatments for ACPA-positive and ACPA-negative RA could be considered as well. This is supported by a study demonstrating that ACPA-positive RA patients initially treated with DMARD mono-therapy displayed increased radiographic joint destruction after 2 years compared to ACPA-negative patients. In contrast, in patients treated with initial combination therapy, no difference between ACPA-positive and ACPA-negative patients with respect to joint destruction was observed [86].

Assuming that ACPA is pathogenic, it is conceivable to hypothesize that ACPA-positive RA patients would benefit from therapies reducing B cells from the circulation. Indeed, it was demonstrated that in refractory RA patients ACPA positivity was predictive for a EULAR response at 24 weeks to rituximab, a therapy to deplete anti-CD20 positive cells. The investigators suggest that ACPA might be used as biomarker to predict responses to rituximab therapy [87]. Since ACPA consist of a collection of citrulline-recognizing antibodies with different reactivities, it was examined if the different fine specificities within the ACPA repertoire could predict response to therapies. Unfortunately, at least for anti-TNF agents, only the presence, but not individual ACPA specificities was associated with response to treatment [88]. Instead, intrinsic differences between the ACPA response and conventional antibody responses, including the disconnection of ACPA avidity maturation and isotype switching [55], and the detection of spontaneous ACPA production by circulating unstimulated plasmablasts/cells [82] might represent targets for novel therapeutic interventions.

Although taking a personalized medicine approach to treat the subgroup of ACPA-positive patients sounds promising, in the large subset of ACPA-negative RA patients, targets to distinguish different subsets are limited, mainly by the absence of robust biomarkers characterizing this manifestation of RA. Therefore, especially in the ACPA-negative group of patients, the identification of anti-CarP antibodies might be clinically useful. Possibly, the population of RA patients is more heterogeneous than only ACPA positive or negative, and anti-CarP-positive RA might represent an additional disease entity with its own genetic and environmental contributions and responses to specific therapies.

Conclusions

Several RA-associated autoantibody systems have been identified. Among these autoantibodies, ACPA exhibit a unique sensitivity for RA with the highest predictive value for RA development and severity. The importance of ACPA in RA diagnosis is emphasized by the inclusion of ACPA status in the 2010 criteria for RA. For the first time, ACPA allowed division of the heterogeneous entity of RA in an ACPA-positive and an ACPA-negative subset, with different genetic and environmental contribution factors. Importantly, ACPA status predicts response to therapies, indicating a biomarker for a personalized medicine approach. Over the past few years, important insight has been gained into the occurrence and aetiopathology of RA by the discovery of anti-citrullinated protein immunity, although the pathogenic potential of ACPA remains elusive. Major alterations in the biomolecular composition of ACPA in health and disease might be the key to discover the mechanisms underlying pathogenicity

of ACPA. Recent evidence suggest a role for ACPA in exacerbating and perpetuating disease by the demonstration that ACPA enhances the formation of citrullinated autoantigens and immunostimulatory molecules containing NETs by neutrophils. Another feed-forward loop driving worse outcome of disease was demonstrated by another group of RA-associated autoantibodies, activating the enzymes that generate targets for ACPA. Although the discovery of ACPA induced a breakthrough in the understanding immunological events underlying ACPA-positive RA, these factors are still poorly understood for ACPA-negative RA. Recently, the identification of anti-CarP antibodies, also present in serum of ACPA-negative RA patients, indicated that even more subgroups of RA in addition to ACPA-positive and ACPA-negative disease might exist. Therefore, it is plausible that in the future, novel autoantibodies systems in RA will be identified, hopefully resulting in unravelling the immunological pathways underlying RA physiopathology, potentially of importance for the development of diagnostic and/or prognostic tools.

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