

## REVIEW

# Citrullinated Proteins in Rheumatoid Arthritis

## Crucial . . . but Not Sufficient!

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### Introduction

Anti-citrullinated protein antibodies (ACPAs) are highly specific for rheumatoid arthritis (RA) and have thus become part of the diagnostic armamentarium in inflammatory arthritis. Circumstantial clinical evidence also suggests that ACPAs may participate in important pathophysiologic processes in RA. This concept has recently been supported by the specific gene-environment interaction between smoking and HLA-DR4 in ACPA-positive but not ACPA-negative patients with RA and by the enhancement of tissue injury by ACPAs in experimental arthritis. In parallel, important progress has been made in the detection and identification of citrullinated proteins as potential targets for ACPAs in inflamed synovium as well as other tissue. However, it becomes increasingly clear that well-defined citrullinated epitopes, rather than the mere presence of citrullinated proteins as such, may be relevant for the induction of ACPAs and, eventually, for the pathogenicity of anticitrulline reactivity. Therefore, defining the clinically relevant citrullinated epitopes and experimen-

tally assessing the requirements for optimal and coordinated immune activation by these epitopes are 2 major challenges in this field.

### From systemic antibodies to synovial targets

In the vast majority of patients with RA, one of the major features is the presence of serum autoantibodies. Besides the well-known rheumatoid factor, the so-called anti-citrullinated protein antibodies have been the subject of increasing scientific and clinical interest. For more than 40 years, these antibodies have been known as antiperinuclear factor, antikeratin antibodies, and antifilaggrin antibodies (1–3). Their commonalities have been evidenced by the crucial finding that they all target epitopes in which arginine residues have been converted to citrulline by the posttranslational action of peptidyl arginine deiminase (PAD) enzymes (4,5). The high specificity for RA (6–8) and the development of citrullinated substrates that allow easy and reliable detection of these autoantibodies (4,5,9,10) have boosted clinical interest in ACPAs as a new diagnostic tool.

Besides this diagnostic application, recent clinical observations have provided some circumstantial evidence that ACPAs may be related to important pathophysiologic processes in RA. First, ACPAs can be found early in the disease course of RA (11–14), even years before the onset of clinical symptoms (15,16). Second, the presence of ACPAs is associated with more severe joint destruction (13,17,18) and greater disease activity (13,18,19), with ACPA positivity at the time of diagnosis being an important predictor of a more aggressive disease course (13,15,17). Third, recent studies suggest that ACPAs define a separate etiologic entity within RA (20,21), and a striking gene-environment interaction between the HLA-DR shared epitope and smoking has

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been described in ACPA-positive but not ACPA-negative patients with RA (22).

Despite this evidence, the role of ACPAs in the pathogenesis of RA is not firmly established. Citrullinated epithelial (pro)filaggrin, the originally described target of ACPAs (3,23,24), is not expressed in the joint, and citrullinated filaggrin-containing epithelial tissue such as skin and buccal mucosa is not involved in RA. These seemingly contradictory findings were put in a new perspective by the demonstration of ACPAs of the IgM isotype (6) and local ACPA production in the inflamed RA joint (25), which strongly suggest a local, antigen-driven B cell response. Collectively, these data supported the hypothesis that distinct citrullinated proteins present in the inflamed synovium are involved in the induction and/or perpetuation of ACPA responses, whereas citrullinated (pro)filaggrin is probably a cross-reactive substrate.

### Identification of citrullinated proteins in RA synovium

The first step in addressing this hypothesis was the demonstration of the presence of citrullinated proteins in RA synovium. Immunoblotting experiments provided the first evidence of the presence of several deiminated proteins in synovial tissue extracts (26,27): focusing on proteins strongly recognized by ACPAs, Serre and coworkers demonstrated the abundant presence of deiminated  $\alpha$ - and  $\beta$ -chains of fibrin and colocalized extracellular/interstitial fibrin deposits with anticitrulline immunostaining. A followup study showed that the citrullinated fibrin is not specific for RA synovium but is also found in spondylarthropathy and inflamed osteoarthritis synovium (26). Other synovial citrullinated proteins have also been detected in these immunoblotting experiments (27). One such protein could be the Sa antigen, which was originally described in extracts from human placenta, spleen, and RA synovium (28). Later, it was shown that the placental Sa antigen corresponds to citrullinated vimentin, and that the RA-specific anti-Sa antibodies recognize citrullinated (but not unmodified) vimentin *in vitro* (29).

Other possible citrullinated synovial proteins include fibronectin,  $\alpha$ -enolase, Epstein-Barr nuclear antigen 1 (EBNA-1), and nuclear proteins. Fibronectin colocalizes with citrulline reactivity on parallel stainings of consecutive sections and on 1-dimensional Western blotting after immunoprecipitation of fibronectin (30); however, because fibronectin can crosslink with fibrin (31,32), these results need to be confirmed by identification of the citrulline-containing epitopes. Alpha-

enolase, which is recognized in its *in vitro* citrullinated form by RA sera, can be found in the same regions of the synovium as anticitrulline staining, but Western blotting of immunoprecipitates from synovial cells failed to confirm *in vivo* citrullination of  $\alpha$ -enolase (33). Similarly, it has been demonstrated that ACPAs bind specifically to the deiminated EBNA-1 protein encoded by Epstein-Barr virus, but the presence of this deiminated protein in RA synovium remains to be demonstrated (34). Finally, immunohistochemical analysis of RA and control synovium also reveals nuclear staining (26,27), suggesting that nuclear proteins such as citrullinated histones might also be present (35,36).

### Citrullinated proteins at other sites of inflammation

The common occurrence of protein citrullination in different forms of joint inflammation raises the question of whether this posttranslational modification is more generally associated with inflammation. Immunohistochemical analysis in RA-associated interstitial fibrosis revealed that citrullinated proteins were present in approximately half of the RA lung samples, whereas lung tissue from normal controls showed almost no staining (37). However, citrullinated proteins were also detected in half of the cases of idiopathic interstitial pneumonia, with no significant difference in the amount or pattern compared with that in RA-associated pneumonia. In contrast to the findings in bronchoalveolar lavage cells (22), no association between smoking status and pulmonary citrullination was observed in lung tissue. A recent study also demonstrated the presence of citrullinated proteins in other inflamed tissues, such as muscle in polymyositis, gut mucosa in Crohn's disease, and tonsils in chronic tonsillitis (38).

The central nervous system (CNS) is of particular interest, because similar to the joint, it is usually isolated from the peripheral immune system and can form the target of a class II major histocompatibility complex (MHC)-restricted autoimmune attack in multiple sclerosis (MS). In chronic and fulminating forms of MS, citrullination of proteins such as myelin basic protein (MBP) (39,40) and glial fibrillary acidic protein (GFAP) (41) is increased in the brain and spinal cord. Because citrullination results in proteins with looser secondary structures (42), deimination of myelin proteins can lead to less compact myelin, which is more rapidly degraded by the proteinase cathepsin D (43,44). This may, in turn, enhance the release of antigenic peptides (45) and thus propagate an autoimmune response, as observed in experimental autoimmune encephalitis in mice (46).

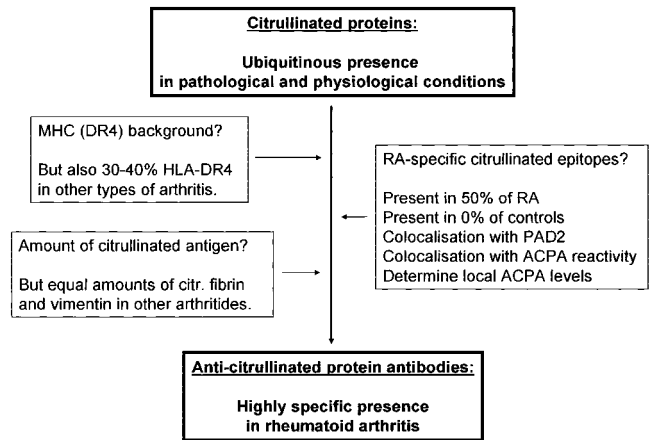
Interestingly, citrullinated proteins are found not only in pathologic conditions but also in the nondiseased CNS. Indeed, 6 of 19 arginines are deiminated in the C8 isoform of MBP (47–49) in the normal developing brain (39), and deiminated GFAP was also evidenced in normal human brain tissue (41). Therefore, the CNS illustrates well that protein deimination occurs in the setting of non-RA inflammation as well as in physiologic situations.

**Is citrullination enough to trigger ACPA responses?**

The widespread presence of citrullinated proteins in a variety of pathologic but also physiologic (36,50,51) conditions clearly indicates that protein deimination as such is not specific for RA synovitis. The more fundamental issue raised here, however, is the striking contrast between the ubiquitous presence of citrullinated proteins and the RA specificity of ACPAs, because ACPAs have, for instance, not been observed in patients with MS (Figure 1). Although it is clear from immunoblotting experiments that not all citrullinated proteins are specifically recognized by ACPAs (27), even the presence of citrullinated proteins with proven antigenic affinity for ACPAs, such as citrullinated (pro)filaggrin in the skin, is not sufficient to initiate the antibody response (52). Moreover, the presence of such deiminated proteins in a suitable inflammatory milieu also is not sufficient to break tolerance, because citrullinated fibrin is present in the inflamed synovium of patients with chronic inflammatory joint diseases other than RA (26). Thus, citrullination of proteins, even under conditions of inflammation, is not sufficient to trigger the RA-specific ACPA response.

**Restriction elements in the ACPA response toward ubiquitous citrullinated proteins**

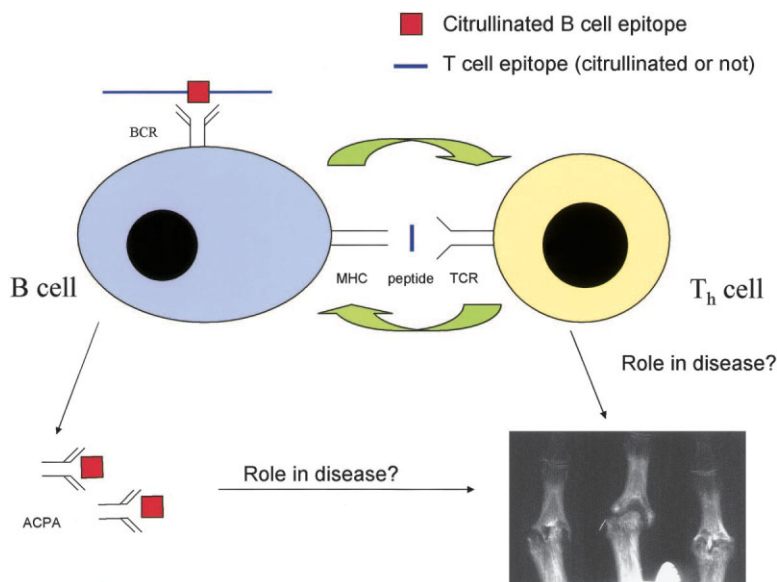
In other conditions in which a highly specific immune response is directed toward a ubiquitous antigen, such as the anti-double-stranded DNA antibodies in systemic lupus erythematosus, experimental models have demonstrated that the balance between tolerance and autoimmunity is controlled by a multitude of complex mechanisms that fall out of the scope of the present review. A similar analysis for ACPA-related autoimmunity is still hampered by the absence of an appropriate experimental model of ACPA induction (53,54), but data on humans have pointed to 2 interesting mechanisms that could be involved in the restriction of the immune response toward citrullinated proteins.



**Figure 1.** Schematic representation of the discrepancy between the ubiquitous presence of citrullinated (citr.) proteins in a variety of pathologic as well as physiologic conditions and the highly rheumatoid arthritis (RA)-specific presence of anti-citrullinated protein antibodies (ACPAs). Potential explanations for this discrepancy include 1) the requirement of an appropriate class II major histocompatibility complex (MHC) background (HLA-DR4 shared epitope), 2) the amount of citrullinated antigen, and 3) the presence of RA-specific citrullinated epitopes. Such RA-specific citrullinated epitopes have been described in inflamed synovium, but their exact biochemical identity is still under investigation. PAD-2 = peptidyl arginine deiminase type 2.

First, it is known from other immune-mediated diseases that not only the presence, but also the amount, of antigen presented to the immune system determines the immune response, by breaking a putative tolerance threshold (55) (Figure 1). Based on an association between *PADI4* polymorphisms and RA in a Japanese population, Suzuki et al proposed that increased PAD-4 messenger RNA (mRNA) stability in RA could lead to an increase in the expression of the deiminating enzyme, which in turn could augment protein citrullination and contribute to breaking the tolerance threshold (56). However, this genetic association is still controversial (57–59), and the hypothesis is challenged by 1) the lack of direct evidence for an increased amount of PAD-4 protein (52) or mRNA in RA, 2) the fact that citrullinated proteins such as fibrin are found in equal amounts in RA and control synovium (26), and 3) the absence of a link between *PADI4* haplotypes, synovial citrullinated proteins, and ACPAs (52,59–61).

A second mechanism that may contribute to the restriction of the antibody response is the requirement for a specific MHC background, as suggested by the higher ACPA levels in HLA shared epitope-positive patients with RA (62–64) and the prominent gene-environment interaction between the shared epitope and



**Figure 2.** Schematic representation of how citrullinated proteins could be involved in the pathogenesis of rheumatoid arthritis. Citrullinated proteins are recognized by autoreactive B cells, which subsequently can differentiate to plasma cells producing anti-citrullinated protein antibodies (ACPAs). Recent data from an experimental model indicate that these ACPAs can enhance arthritis (86), but it remains to be proven that they can also induce disease. The linkage of ACPA levels with the HLA-DR shared epitope and the isotype of the ACPA strongly suggest that autoreactive B cells require appropriate T cell help. However, this requirement, as well as the exact specificity of these T cells (toward citrullinated or noncitrullinated epitopes of the citrullinated protein?), remain to be formally demonstrated. Insights from other models of autoimmunity suggest that coordinated activation of both autoreactive B lymphocytes and T lymphocytes may be necessary for development of pathology. Whereas circumstantial clinical evidence supports this hypothesis, the development of new experimental models for citrulline-related autoimmunity will be crucial to address these issues formally. BCR = B cell receptor; MHC = major histocompatibility complex; TCR = T cell receptor; T<sub>h</sub> = T helper.

smoking in ACPA-positive but not ACPA-negative patients with RA (22). One caveat here is that high titers of ACPA develop in some patients with RA who lack the HLA shared epitope, but this could be explained by the presence of other permissive MHC molecules such as HLA-DRB1\*1501 (65). A more important drawback is that in patients with spondylarthritis and patients with psoriatic arthritis, who have citrullinated proteins such as deiminated fibrin in their synovial membrane (26), ACPAs do not develop, despite the presence of the HLA-DR shared epitope in almost half of such patients (66). Therefore, it is clear that the HLA-DR shared epitope is involved in the ACPA response, but that the MHC background alone does not explain the specificity of ACPAs for RA.

### Defining autoepitopes

The link between ACPAs and class II MHC strongly suggests that the presentation of specific peptides by HLA-DR to CD4<sup>+</sup> T lymphocytes and the resulting T cell activation play an important role in the ACPA response (Figure 2). The *in vitro* conversion of arginine into citrulline at the peptide side-chain position interacting with the HLA shared epitope significantly increased peptide-MHC affinity and led to the activation of CD4<sup>+</sup> T cells in DR4-IE-transgenic mice (67). However, the higher affinity for HLA-DR4 was demonstrated with only 1 citrullinated epitope of vimentin and could not be reproduced using a wide panel of peptides derived from fibrin (68). Moreover, T cell responses were directed toward both citrullinated and noncitrullinated

nated fibrin epitopes and were detected in RA as well as in control arthritides (68).

Whereas further research is needed to resolve the discrepancies with regard to HLA-DR4 binding, these data point toward 2 important issues. First, activation of T lymphocytes could be a crucial step in the loss of B cell tolerance toward citrullinated proteins. Second, whereas citrullination is crucial for B cell/antibody reactivity, it is uncertain whether citrullination is required for the T cell epitope in order to provide adequate T cell help for the humoral autoimmune response (Figure 2). A citrullinated protein recognized by ACPAs and containing the correct deiminated B cell epitopes but lacking appropriate T cell epitopes (deiminated or not) may fail to induce a strong ACPA response. Indeed, different immunogenic peptides yield quantitatively and qualitatively different autoimmune responses, a phenomenon that appears to be dependent on the antigen-specific interaction between autoreactive T cells and B cells rather than on the amplitude of the lymphocyte activation as such (69–72). So-called autoepitopes appear to have the unique property of promoting self-perpetuating interactions between B lymphocytes and T lymphocytes and reciprocal T cell–B cell diversification in a variety of autoimmune diseases, including systemic lupus erythematosus, MS, and celiac disease (73–77). Thus, the ability to induce a strong and diversified autoimmune response is a specific property of a well-defined autoepitope. In the case of anticitrulline reactivity in RA, these autoimmune responses may vary widely between different citrullinated epitopes, and the presence of such specific autoepitopes, rather than citrullination of proteins as such, may be crucial to initiate the ACPA response.

### Citrullinated B cell epitopes

Although we lack the appropriate experimental models with which to test these concepts relating to ACPA responses in RA, it is well known that in vivo ACPAs are directed against various citrullinated epitopes in which not only the citrulline residue, but also the flanking amino acids, make up the antibody recognition site (4,5). This is illustrated by the fact that different citrullinated substrates yield different ACPA sensitivities and specificities for RA when used in the same patient cohorts (63,78). The ACPAs produced in vitro are also epitope specific and recognize a distinct subset of citrullinated peptides, as illustrated by the different staining patterns in an immunohistochemistry study using different anticitrulline reagents (79), and by the fact that monoclonal antibodies raised against in

vitro citrullinated proteins only rarely cross-react with other citrullinated proteins (Baeten B: unpublished observations). As is the case for myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and MS (80), the citrullinated epitopes may even be conformation dependent and thus lose their reactivity under certain in vitro conditions.

In most of the above-mentioned studies, researchers used the commercially available anti-modified citrulline antibody for the detection of citrullinated proteins in synovial tissue. This antibody detects all citrullinated proteins irrespective of the amino acid context and the structural conformation, because the chemically modified citrulline is voluminous and shields neighboring amino acids (81). As indicated, however, the challenge at this point is not to identify all synovial citrullinated proteins nor even to identify those citrullinated proteins that bind ACPAs with high affinity and specificity, but rather to identify those selected deiminated autoepitopes that are responsible for the specific induction of ACPAs in RA. One way to approach this complex problem is to screen defined citrullinated epitopes for specific disease associations in human RA.

### RA-specific citrullinated epitopes as an alternative hypothesis

We previously demonstrated the presence of distinct intracellular citrullinated proteins in RA synovium, using a rabbit polyclonal antibody raised against poly-L-citrulline (82). Several followup studies, including a study with a mouse monoclonal antibody, confirmed these findings and ruled out the detection of free citrulline (52,66,83,84). Four observations point to particular relevance of the detected citrullinated targets for RA: 1) they are highly specific for RA, being present in approximately half of the RA synovia but in none of the controls; 2) they colocalize with PAD-2, which, in contrast to PAD-4, appears to be overexpressed in RA synovium and could thus at least partially explain the RA specificity of the intracellular citrullinated proteins (52); 3) they also colocalize with ACPA reactivity in RA synovium, suggesting that they can be recognized by the autoantibodies (82); and 4) in contrast to other citrullinated proteins (26,79), the presence of the RA-specific intracellular citrullinated proteins determines the local production of ACPAs in an HLA-DR shared epitope-restricted manner (52,60) (Figure 1). Although the biochemical identity of these proteins is still under investigation, these data suggest that despite the ubiquitous presence of citrullinated proteins in physiologic and

pathologic conditions, the restricted presence of well-defined citrullinated epitopes in RA synovium may contribute to the highly specific induction of ACPAs in this disease. These data also suggest that PAD-2, rather than PAD-4, may be relevant for the deimination of these well-defined epitopes. In contrast to *PADI4*, functional haplotypes for *PADI2* have not yet been investigated.

### Implications for the pathophysiology of RA

If well-defined citrullinated autoepitopes, rather than merely ubiquitous citrullinated proteins, are relevant for the induction of ACPAs, the identification of these epitopes could provide crucial insights into the pathophysiology of RA. Indeed, it remains an open question whether anticitrulline autoreactivity in RA is primarily involved in the pathogenesis, results in secondary enhancement and/or perpetuation of inflammation, or is simply a bystander phenomenon.

Although original reports failed to demonstrate specific anticitrulline responses in different arthritis models (53,54), there is now emerging evidence that ACPAs may contribute to the enhancement of arthritis. Immunization with deiminated collagen rather than native collagen slightly aggravates collagen-induced arthritis (CIA) in mice (85). More importantly, in mice tolerized by intravenous administration of a citrulline-modified peptide before collagen immunization, disease severity was reduced compared with that in mice treated with a control peptide and ovalbumin, and passive transfer of monoclonal antibodies specific for citrullinated fibrinogen in combination with anti-type II collagen antibodies enhanced disease severity compared with that in mice receiving anti-type II collagen antibodies alone (86). Although further control experiments with antibodies to noncitrullinated fibrinogen should confirm the specificity of this observation, these data demonstrate for the first time that ACPAs can enhance tissue injury in CIA. In contrast, however, antibodies against citrullinated fibrinogen failed to aggravate adjuvant-induced arthritis in Lewis rats (87).

Whereas these emerging data show that ACPAs could enhance disease severity in arthritis models, there is still no evidence for a primary role of citrullinated proteins and/or ACPAs in the induction of the arthritis. Indeed, immunization with citrullinated proteins such as fibrinogen failed to induce arthritis, even in HLA-DR4-transgenic animals, although they were clearly immunogenic (87,88). Accordingly, transfer of ACPAs alone failed to induce arthritis in DBA/1 mice (86). Interpret-

ing these data from the perspective of the recent findings in humans, it is not surprising that citrullinated proteins as such are not pathogenic. Consistent with the observations of RA-specific citrullinated epitopes in human synovium (52,82) and of the critical quantitative and qualitative impact of specific epitopes on the immune response in other autoimmune disease models (69), it is likely that well-defined citrullinated autoepitopes on a background allowing optimal and coordinated immune reactivity will be needed in order to assess the real pathogenic potential of anticitrulline immunoreactivity (Figure 2).

### Conclusions

Increasing circumstantial evidence from clinical research supports the concept that ACPAs define a separate pathophysiologic entity within RA, and that citrullinated proteins may be involved in the induction of this process. Major progress has been made in the detection and identification of citrullinated proteins as potential targets for ACPAs in inflamed synovium as well as other tissue. Importantly, however, it becomes clear that well-defined citrullinated epitopes, rather than the mere presence of citrullinated proteins as such, may be relevant for the induction of ACPAs and, eventually, for the pathogenicity of anticitrulline reactivity. Therefore, defining the clinically relevant citrullinated epitopes and experimentally assessing the requirements for optimal and coordinated immune activation by these epitopes are 2 major challenges in this field.

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