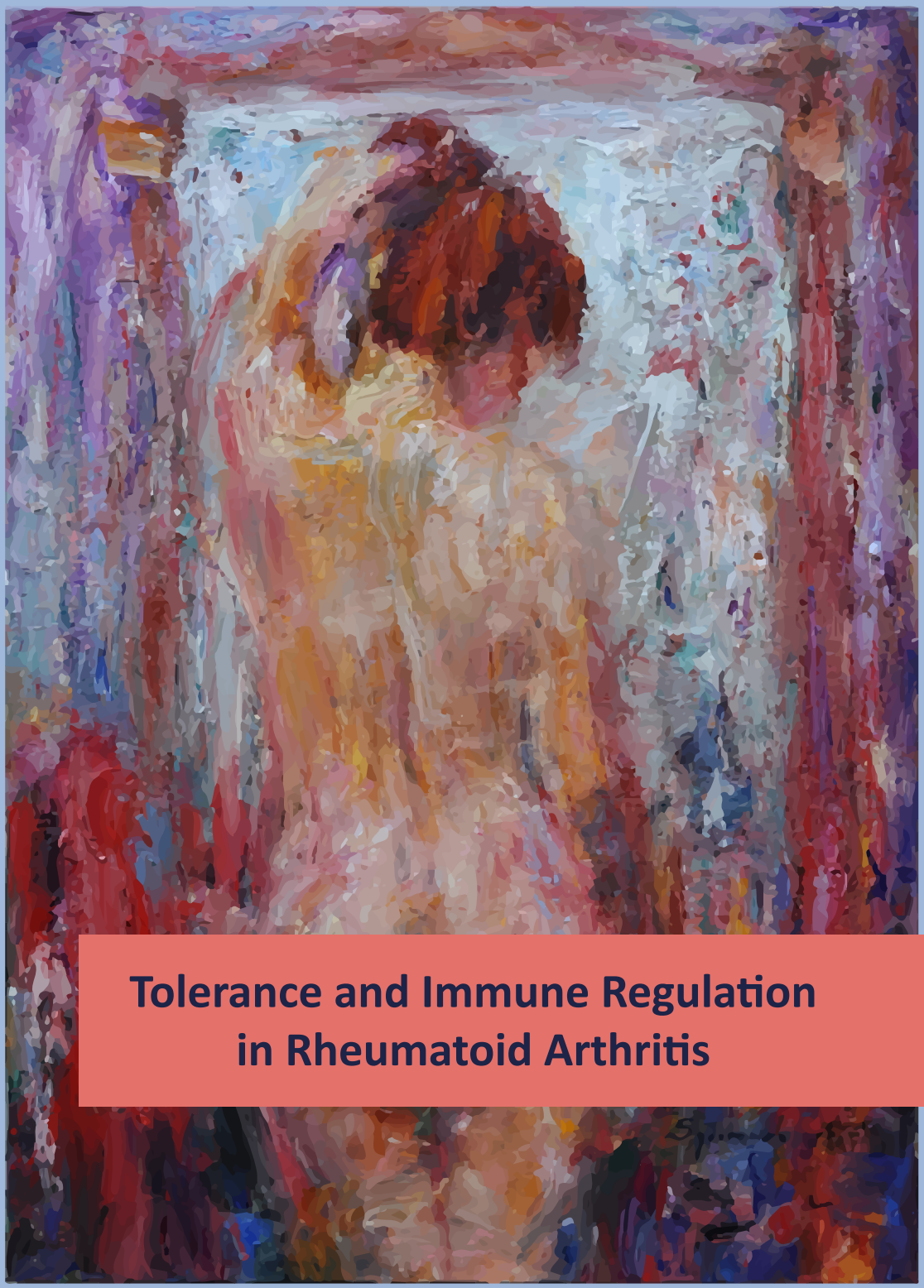
Tolerance and immune regulation in rheumatoid arthritis

Dekkers, J.S.



Tolerance and Immune Regulation in Rheumatoid Arthritis

Jacqueline S. Dekkers

Tolerance and Immune Regulation in Rheumatoid Arthritis

Jacqueline Stephanie Dekkers

The studies described in this thesis were performed at the Department of Rheumatology at the Leiden University Medical Centre, Leiden, the Netherlands

ISBN: 978-94-028-1644-0

The printing of this thesis was financially supported by: Universiteitsbibliotheek Leiden, Chip Soft, UCB and Pfizer.

Cover design: *"At the mirror"*, oil on linen by Misha Sydorenko, 2016. In private collection.

Tolerance and Immune Regulation in Rheumatoid Arthritis

Proefschrift

Ter verkrijging van de graad Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof.mr.C.J.J.M. Stolker,

volgens besluit van het College voor promoties

te verdedigen op dinsdag 1 oktober 2019

Klokke 15:00 uur

door

Jacqueline Stephanie Dekkers

Geboren te Rotterdam

in 1989

Promotores

Prof.dr. R.E.M. Toes

Prof.dr. T.W.J. Huizinga

Copromotor

Dr. D. van der Woude

Promotiecommissie

Prof.dr. G. Kloppenburg

Prof.dr. F.A. Ossendorp

Dr. E. Lubberts, Erasmus MC, Rotterdam

Dr. S.W. Tas, Amsterdam UMC, Amsterdam

Content

Chapter 1 Introduction and outline 9

PART I. ROLE OF PROTEIN MODIFICATIONS ON AUTOIMMUNITY

Chapter 2 The role of anticitrullinated protein antibodies in the early stages of rheumatoid arthritis. 27
Current Opinion in Rheumatology 2016 May;(3):275-81.

Chapter 3 Breach of autoreactive B-cell tolerance by post-translationally modified foreign proteins. 43
Annals of the Rheumatic Diseases. 2017 Aug;76(8):1449-1457

Chapter 4 Carbamylation of self-antigens facilitates a breach in T-cell tolerance 77

Chapter 5 Different classes of Anti-Modified Protein Antibodies are induced upon exposure to antigens expressing one type of modification. 91
Annals of the Rheumatic Diseases. 2019 Jul;78(7):908-916

PART II. AUTOIMMUNITY IN EARLY DISEASE

Chapter 6 Autoantibody status is not associated with early treatment response to first-line methotrexate in patients with early rheumatoid arthritis 121
Rheumatology (Oxford). 2019 Jan 1;58(1):149-153.

Chapter 7 Possibilities for preventive treatment in rheumatoid arthritis? Lessons from experimental animal models of arthritis: a systematic literature review and meta-analysis. 139
Annals of the Rheumatic Diseases. 2017 Feb;76(2):458-467.

Chapter 8 Periodontal infection and induction of autoimmunity in rheumatoid arthritis: *Aggregatibacter actinomycetem comitans*-induced hypercitrullination 173
Science Translational Medicine 2018 Mar 21;10(433).

Chapter 9 Discussion and general summary 181

Chapter 10	Nederlandse samenvatting	193
Appendices	List of publications	201
	Curriculum Vitae	203
	Dankwoord	205

Chapter 1

Introduction and outline

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by synovial inflammation, autoantibody production and destruction of cartilage and bone (1). Classically, the disease affects the small joints of the hands and feet in a symmetrical pattern. As RA is a systemic disease, extra-articular manifestations can be present in almost any system of the body e.g. the skin, heart, lungs and blood vessels (1). The diagnosis of RA is predominantly a clinical one, as a history of progressive joint swelling, stiffness and increased pain after a period of inactivity is indicative of an inflammatory joint disease. Early diagnosis and timely initiation of disease-modifying antirheumatic drugs, has been shown to have a favourable effect on the course of disease (2-4). However, timely diagnosis remains a challenge as classical signs of structural changes may be missing in early disease and subtle synovitis may escape notice during clinical examination.

Over the past few years, research in the field of RA has focused on the earliest stages of disease, leading to the discovery that circulating autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) which precede the clinical onset of disease (5-7). ACPA are highly specific for RA (8) and the presence of the self-reactive antibodies reflects the complexity of disease. As RA seems to be a highly heterogenous disease, classification criteria have been developed with the aim to identify more homogenous patient groups to facilitate comparison with international studies. Using a quantitative scoring system patients can be classified based on scores obtained from; joint involvement, serologic markers, inflammatory markers and duration of symptoms. Although RA is a clinical diagnosis and diagnostic criteria for RA do not exist, classification criteria have been developed to arrive at homogeneous inclusion in clinical trials. The 1987 American College of Rheumatology (ACR) classification criteria for RA were not very well suited for the inclusion of recent onset RA as they rely on the expression of clinical symptoms, a feature of established RA (9). These findings led to the inclusion of an additional early serological marker, ACPA, to the current 2010 American College of Rheumatology/European League Against Rheumatism (2010 ACR/EULAR criteria) classification criteria for rheumatoid arthritis (10), (Table 1).

Although biomarkers provide valuable information to identify patients at an early disease phase, interpretation within the clinical context remains vital. For example, if the prior probability of RA is relatively low, such as in patients in primary care who have only knee pain or those who meet no other ACR criteria, measuring antibodies reactive to cyclic citrullinated peptide (CCP) alone seems to be a reasonable strategy that avoids too many false-positive results. If however, the prior probability of RA is relatively high, such as in patients seen in rheumatology clinics, measuring both autoantibodies seems to be a reasonable strategy that avoids missing potentially treatable patients.

Table 1. ACR/EULAR 2010 classification criteria for rheumatoid arthritis.

Joint involvement	**Score**
1 large joint	0
2-10 large joints	1
1-3 small joints (large joints not counted)	2
4-10 small joints	3
>10 joints (at least one small joint)	5
Serology	
Negative RF and negative ACPA status	0
Low-positive RF or low-positive ACPA	2
High-positive or high-positive ACPA	3
Acute phase-reactants	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
Duration of symptoms	
<6 weeks	0
≥ 6 weeks	1

A score ≥ 6 is the cut point for rheumatoid arthritis. Patients can also be classified as having rheumatoid arthritis in the presence of 1) erosive disease, 2) long-standing disease according to the previous classification criteria. Target population: patients who have at least one joint with definite clinical synovitis, not better explained by another disease. Joint involvement: any swollen or tender joint on examination. Large joint: shoulders, elbows, hips, knees and ankles. Small joint: joints in the hands, writs and feet. Negative serology: below or equal to upper limited of normal (ULN). Low-positive serology: higher than ULN, less then 3 times ULN. High-positive serology: more than 3 times ULN. Acute phase reactants: according to local standards. Duration of symptoms: reported by the patient or symptoms of synovitis at the time of assessment.
RF= rheumatoid factor, ACPA=anti-citrullinated protein antibodies, CRP= C-reactive protein, ESR= erythrocyte sedimentation rate (10).

Autoantibodies in RA

The first RA associated autoantibody was RF which was identified in 1940 (11). RFs are antibodies that bind immunoglobulin (Ig)G and recognizes epitopes in the fragment crystallizable (Fc) region, which is responsible for effector functions like complement binding (12). Although usually measured as IgM-RF, RF activity can also be found in other subclasses of Ig (13). RF can be detected in 50-90% of RA patients, but are also found in the sera of patients with other connective tissue diseases, patients with infectious diseases and elderly healthy individuals (14). RF was the most valuable diagnostic marker for RA before

the discovery of ACPA and is still part of the 2010 ACR/EURLAR classification criteria. The presence of RF and ACPA represents an important early biomarker as these autoantibodies can be detected early in the disease course and can precede the clinical onset of disease (5-7). RF an ACPA can be found in sera of patients a median of 5 years before onset of clinical symptoms (6). Subjects with arthralgia which are seropositive for these autoantibodies have an approximately 30% chance of developing RA within one year (15). These findings highlight an important role for autoantibodies in the preclinical phase of RA which precedes the onset clinical signs and symptoms. Most research on the role of autoantibodies in RA has focused on ACPA, which are directed against citrullinated proteins. In the past several years it has become clear that the autoantibody response in RA extends to several other modified proteins, such as proteins modified by carbamylation and acetylation. As all these auto-antibodies recognize post-translationally modified proteins, these antibodies are collectively called anti-modified protein antibodies (AMPA). A variety of AMPAs against different protein modifications (anti-citrullinated (16), -carbamylated (17) and -acetylated protein antibodies (18)) have now been described in RA suggesting a shared common 'developmental' basis.

Post-translational protein modifications

Post-translational protein modification refers to the covalent and generally enzymatic modification of proteins following biosynthesis. Protein modifications can exist in different forms e.g. addition of small chemical groups, fatty acids or sugar chains, and can occur on the amino acids side chains or at the protein's C- or N- termini (19). Most post-translational modifications can modulate the protein conformation, function, activity, stability and/or location of a protein. For example, phosphorylation is a very general post-translational protein modification involved in the regulation of enzyme activity.

AMPA responses directed against post-translational modified proteins are highly specific for RA as they are, by and large, not found in other auto-immune or inflammatory diseases (20). As many proteins in the joint are long-lived especially extra-cellular matrix proteins expressed in the cartilage, it is not surprising that modified proteins can be found at higher levels in the joint compartment (21). Interestingly, the increased expression of modified proteins in the joints and cartilage might contribute to the production of AMPAs and possibly explain why AMPAs are associated with rheumatic diseases especially RA. As these post-translational modified proteins are prolongedly expressed and possibly more exposed by extracellular expression in the synovial compartment and cartilage, AMPA responses can contribute to increased susceptibility and risk of chronic inflammatory responses of the joints (20). For example, plasma proteins which carry post-translational modifications are readily recognised by AMPAs which can result in immune complex formation, complement

activation and subsequent clearance of the circulation. By contrast, modified matrix molecules recognised by AMPAs also induce immune complex formation and complement formation, however as these proteins are expressed in the synovial compartment they are immobile and these immune responses do not result in clearance of the modified proteins (22). This enables a prolonged process of complement activation, attraction of immune cells and release of mediators like myeloperoxidase (MPO) which stimulates local inflammation and protein modification contributing to chronic inflammatory responses.

Anti-citrullinated protein antibodies

Citrullination is a post-translational modification of proteins, in which a positively charged peptidylarginine residue is converted into a neutral peptidylcitrulline (Figure 1) (8). The conversion is catalysed by the calcium dependent peptidylarginine deiminases (PAD) enzyme. This modification takes place during a variety of biological processes including inflammation. ACPA are a hallmark of RA as these autoantibodies can be present many years prior to disease onset, and their presence is associated with an increased risk of developing RA (6, 15, 23). In the pre-RA phase the ACPA response matures which is characterised by an increase in isotype diversity, the range of epitopes recognized and the levels of antibodies (24). Both the presence of ACPA as well as the extent of the ACPA response are associated with clinical outcomes (25). ACPA positive patients respond different to therapy compared to ACPA negative patients (23, 24), have an more destructive disease course (26) and have an increased risk of relapse after tapering of disease-modifying antirheumatic drug (DMARD) therapy (27). The presence of ACPA can be detected by several commercially available laboratory tests. The first assay was based om the artificial CCP. Antibodies reactive to this assay are referred to as anti-CCP antibodies. While ACPA and RF are highly prevalent in RA they can also be identified in a small percentage of healthy individuals. The anti-CCP assay has a moderate sensitivity for RA (67%), comparable to IgM-RF (69%), and a very high specificity (95%) at the optimal cut-off value, whereas the sensitivity of IgM-RF is reported to be around 85% (16, 28).

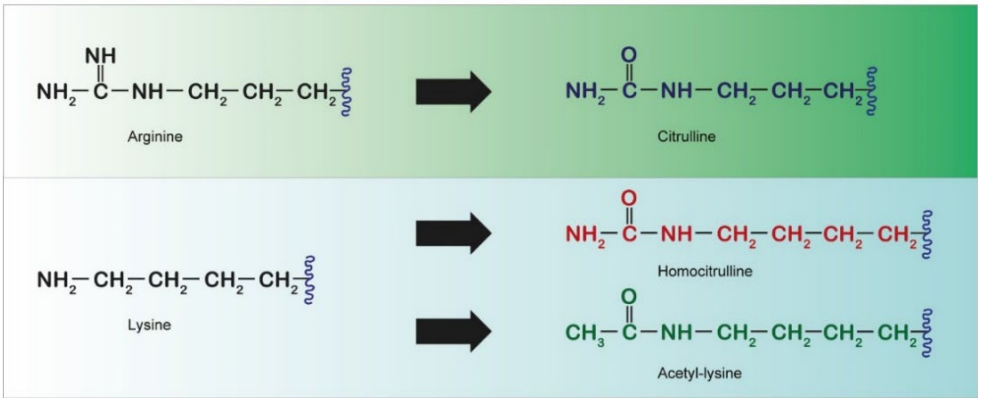


Figure 1. Chemical structures of post-translational protein modifications. Conversion of arginine into citrulline by PAD enzymes. Chemical modification of lysine into homocitrulline through cyanate. Enzymatic modification of lysine to acetyl-lysine by Acetyl-CoA.

Anti-carbamylated protein antibodies

When compared to ACPA, anti-carbamylated protein antibodies (anti-CarP antibodies) were discovered more recently (17). Anti-CarP antibodies target proteins that contain homocitrullines, also referred to as carbamylated proteins. Carbamylation is a chemical modification which occurs in the presence of cyanate. The formation of cyanate may result in a reaction with any accessible primary amine, including that of the lysine side chain and the amine at the N-terminus of polypeptides (29). The product formed by carbamylation, peptidyl-homocitrulline, highly resembles peptidyl-citrulline but contains one additional CH_2 group (Figure 1). Conversion of lysine into homocitrulline, similar to the conversion of arginine into citrulline, results in the loss of a positive charge which influences protein structure and function. Cyanate can be increased upon chronic inflammation (30) due to the conversion of thiocyanate into cyanate by myeloperoxidase, atherosclerosis (31) and smoking (32). Cyanate, a dissociation product of urea, is also increased in patients with renal failure as urea accumulates with decline of renal function (33).

The observation that the chemical structure of homocitrulline strongly resembles citrulline inspired experiments to analyse whether autoantibodies exist that target carbamylated proteins as well. In the first experiments to identify anti-CarP antibodies, carbamylated fetal calf serum (FCS) was used as a model antigen (17). Subsequent studies revealed that anti-CarP antibodies recognize carbamylated self-antigens like human fibrinogen(34), demonstrating that anti-CarP antibodies are autoantibodies, able to recognize modified self-protein. Using molecularly-defined peptides (containing carbamylated epitopes) anti-CarP antibodies were found to be reactive to peptides from α-enolase (35), collagen (36),

fillagrin (37) and vimentin (18). These studies revealed that protein modification is crucial for antibody recognition. Although several human proteins are currently used in the laboratory for the detection of anti-CarP antibody response, there is little information available on the specific nature and location of carbamylated proteins present in the (inflamed) joint.

Recent clinical studies revealed that anti-CarP antibodies are specific for RA and can be found in 45% of patients with early RA (17, 34, 36, 38, 39). Similar to ACPA, anti-CarP antibodies and can be identified in sera of patients years before onset of clinical symptoms (39-41). In addition, anti-CarP antibodies are associated with bone erosions, disability and mortality, independent of anti-CCP antibodies (17, 38, 39, 41). As anti-CarP antibodies occur in 10-20% of RA patients seronegative for ACPA (17, 38, 40), anti-CarP antibodies are thought to represent an additional serological marker distinct from ACPA. The sensitivity of the anti-CarP assay is reported to be moderate (44%) and with a high sensitivity (89%) (42). With the arrival of anti-CarP antibodies as a serological marker, it is intriguing whether these autoantibodies could contribute to the early identification and classification of patients with RA. Although anti-CarP antibodies are distinct from ACPAs, it has been described that some ACPA can bind peptides or proteins containing homocitrulline (18, 34, 35). However, the degree of cross-reactivity of ACPA for homocitrullinated proteins varies between studies. These discrepancies may be a result of proteins that contain different numbers and locations of homocitrulline and citrulline. Current studies using the same peptide backbone are inconclusive as the affinity of antibodies to citrulline and homocitrullinated peptides was not investigated (18, 35, 36, 43). However, the relative cross-reactivity between ACPA and anti-CarP antibodies suggests that these autoantibodies may originate from the same B-cell population.

Interestingly, anti-CarP antibodies can be detected in sera of mice with collagen-induced arthritis, even in the absence of ACPAs (44). Studies revealed that anti-CarP antibodies can be generated by immunization of rodents and rabbits with carbamylated proteins (37, 45). In rabbits, the induced response was partially cross-reactive. Comparison of different mouse and rat models of arthritis revealed that anti-CarP antibodies are only present in models that require active immunisation (46). Time course experiments in collagen-induced arthritis models indicate that the presence of anti-CarP antibodies precedes the onset of clinical symptoms (44), an observation similar to findings in patients with RA. These data suggest that anti-CarP antibodies or B-cell may play a pathogenic role in the development of arthritis.

Anti-acetylated protein antibodies

The most recent identified AMPA responses in RA are the anti-acetylated protein antibodies, directed against the post-translationally modification lysine by acetylation. Anti-acetylated protein antibodies have been described in approximately 40% of RA patients, mainly in the ACPA positive group (18). The identification of another AMPA response in RA provides interesting new insights in the pathophysiology of RA.

Lysine acetylation is a common post-translational modification and is involved in various biological processes such as chromatin remodelling, activation of transcription factors and regulation of metabolic enzymes. Protein lysine acetylation refers to the post-translational addition of an acetyl group to the ε-amino group of the side chain of a lysine residue (47). Acetylated lysine resembles homocitrulline except for the side-chain terminal amine, which is replaced by a methyl group (Figure 1). Multiple acetyltransferases are responsible for lysine acetylation and the first lysine acetylation was discovered on histones (48). Imbalance in histone acetylation has been found to change the chromatin structure and is associated with dysregulation of genes involved in cell-proliferation, differentiation and apoptosis (49-51). Protein acetylation has historically been considered a predominant eukaryotic phenomenon. Recent evidence, however, shows that also many bacterial species are able to acetylate proteins (52), including bacteria postulated as link between periodontal infection and RA (53, 54). These bacteria include *Porphyromonas Gingivalis* as well as *Aggregatibacter Actinomycetemcomitans*. It has been hypothesised that bacterial acetylation could play a role in the development of AMPA responses. Epidemiological data suggest that periodontal disease is more common in RA (35%) compared to controls (37%) (55). Interestingly, citrullinated, carbamylated and malondialdehyde-acetyladehyde (MAA) adduct modified proteins have been found in inflamed periodontal tissues (56). It has been shown that MAA adduct formation, as a result of oxidative stress, are increased in RA patients (57). Current studies reveal that the AMPA response is diverse and heterogenous between patients, but also point at the possibility of a shared developmental basis and role in pathophysiology.

Outline of this thesis

The general aim of this thesis was to elucidate the immune regulation and breach of tolerance towards modified proteins in RA. AMPA responses are a hallmark of disease and are implicated in the pathogenesis of RA. Recent studies have shown that these autoantibodies can serve as diagnostic and prognostic biomarkers.

The studies described in **chapter 2** describe the current evidence supporting the notion that autoimmunity against citrullinated proteins is already present at a preclinical phase of RA and how the ACPA response matures over time. Recent clinical studies have demonstrated that effective treatment of arthritis can lead to reduced levels of ACPA or a change in composition of ACPA. Many autoimmune diseases including RA, are characterized by the production of antibodies that can bind self-antigens. Animal studies have shown that the immunogenicity of proteins is enhanced upon protein modification (58-60). However, how these self-reactive B cells escape negative selection in the thymus is not known. To understand how autoreactive B cell responses are generated to post-translational modified proteins we hypothesised that exposure to carbamylated self-antigens is sufficient for a breach of immunological tolerance. The studies described in **chapter 3** show that exposure of mice to carbamylated self-antigens can lead to the formation of anti-CarP antibodies. Our observations reveal that not only carbamylation of self- but also foreign proteins is sufficient to induce self-reactive AMPA responses. In line with these findings, it was recently shown that homocitrulline containing peptides are also immunogenic in SE-expressing DR4tg mice and lead to T and B cell responses directed to carbamylated antigens (61).

Activation of naïve B cells by T cell dependent signals is characterised by the formation of germinal centers. Activated T-cells provide a stimulatory signal to B cells via CD40L-CD40 interaction (62) which subsequently leads to B cell proliferation and differentiation. In **chapter 4** we analysed whether carbamylation of an antigen can result in the generation of neo-epitopes and the subsequent induction of a breach tolerance at T cell level towards self-antigens. Our results indicate that carbamylated self-proteins are sufficient for a breach of immunological tolerance and are able to trigger primary immune responses, including autoantibody formation, T cell activation and cytokine production. In our studies we describe the concept that post-translational modification of self-proteins can create “new” antigens for which immune tolerance does not yet exist. To better understand the origin of AMPA responses and development of AMPA-producing B cells we immunized mice with different post-translational modified proteins and studied the induced humoral responses. These studies are described in **chapter 5** and indicate that exposure to a particular class of modified proteins (carbamylated or acetylated) can induce an immune response against another class of modified proteins as well. Likewise, AMPA from RA-patients purified against one PTM can recognize different classes of PTMs. These findings are important as

they indicate that the different AMPA-responses observed in RA-patients can be derived from the same inciting antigen(s) carrying only one particular modification.

Since the discovery of autoantibodies in RA it has been hypothesized that patients may benefit from treatment tailored to "autoantibody status". As methotrexate is the most widely used anti-rheumatic drug in clinical practice, it would be important to know whether the presence of autoantibodies is associated with better treatment response. In **chapter 6** we show that autoantibody status is not associated with early remission in newly diagnosed RA-patients receiving methotrexate. The results from our study in 1826 RA-patients from the METEOR database (an international rheumatoid arthritis registry) indicate that methotrexate is effective as initial treatment strategy regardless of autoantibody status.

The studies described in **chapter 7** focus on the preclinical disease phase of RA as it has been thought that treatment initiation in pre-arthritis stages might result in an improved treatment efficacy and possibly the prevention of a full-blown disease. In animal studies with the collagen-induced arthritis and adjuvant-induced arthritis model we aimed to study whether treatment initiation in a prophylactic or pre-arthritis setting could reveal differences in treatment efficacy between antirheumatic drugs. In this systematic literature study we describe the evidence that both prophylactic and pre-arthritis treatment strategies can lead to a significant reduction of arthritis severity scores, which hints at a possibility for preventive treatment strategies in RA.

Several studies have shown a clinical and epidemiological association between periodontitis and RA (63). Periodontal disease is characterized by gingivitis and a chronic inflammatory process of the bone. *Aggregatibacter actinomycetemcomitans* (Aa), a specific periodontitis-associated bacterium and its lytic toxin (Leukotoxin A or LtxA) were hypothesized to be involved in the initiation of ACPA responses in genetically predisposed individuals (54). In **chapter 8** we studied whether RA patients of Leiden Early Arthritis Clinic had an increased prevalence of Aa, measured by the presence of anti-LtxA antibodies. Furthermore, we aimed to replicate the finding that the association between human leukocyte antigens shared epitope (HLA SE) alleles and ACPA-positive RA is limited to the anti-LtxA-positive subset of patients. Using sera of arthritis patients we found that the presence of anti-LtxA antibodies was not specifically associated with RA. In addition, no association with the presence of ACPA or HLA SE alleles among RA patients was found. Finally, **chapter 9** discusses and summarizes the results of the studies described in this manuscript and describes possible directions for future research.

References

1. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med. 2011;365(23):2205-19.
2. Lard LR, Visser H, Speyer I, vander Horst-Bruinsma IE, Zwinderman AH, Breedveld FC, et al. Early versus delayed treatment in patients with recent-onset rheumatoid arthritis: comparison of two cohorts who received different treatment strategies. Am J Med. 2001;111(6):446-51.
3. Nell VP, Machold KP, Eberl G, Stamm TA, Uffmann M, Smolen JS. Benefit of very early referral and very early therapy with disease-modifying anti-rheumatic drugs in patients with early rheumatoid arthritis. Rheumatology (Oxford). 2004;43(7):906-14.
4. van der Linden MP, le Cessie S, Raza K, van der Woude D, Knevel R, Huizinga TW, et al. Long-term impact of delay in assessment of patients with early arthritis. Arthritis Rheum. 2010;62(12):3537-46.
5. Berglin E, Padyukov L, Sundin U, Hallmans G, Stenlund H, Van Venrooij WJ, et al. A combination of autoantibodies to cyclic citrullinated peptide (CCP) and HLA-DRB1 locus antigens is strongly associated with future onset of rheumatoid arthritis. Arthritis Res Ther. 2004;6(4):R303-8.
6. Nielen MM, van Schaardenburg D, Reesink HW, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MH, et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum. 2004;50(2):380-6.
7. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum. 2003;48(10):2741-9.
8. van Venrooij WJ, van Beers JJ, Pruijn GJ. Anti-CCP Antibody, a Marker for the Early Detection of Rheumatoid Arthritis. Ann N Y Acad Sci. 2008;1143:268-85.
9. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988;31(3):315-24.
10. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010;62(9):2569-81.
11. Waaler E. On the occurrence of a factor in human serum activating the specific agglutintion of sheep blood corpuscles. 1939. Apmis. 2007;115(5):422-38; discussion 39.
12. Moore TL, Dorner RW. Rheumatoid factors. Clin Biochem. 1993;26(2):75-84.
13. Tarkowski A, Nilsson LA. Isotype-specific measurement of rheumatoid factor with reference to clinical features of rheumatoid arthritis. J Clin Lab Immunol. 1983;12(3):129-35.
14. Shmerling RH, Delbanco TL. The rheumatoid factor: an analysis of clinical utility. Am J Med. 1991;91(5):528-34.
15. van de Stadt LA, van der Horst AR, de Koning MH, Bos WH, Wolbink GJ, van de Stadt RJ, et al. The extent of the anti-citrullinated protein antibody repertoire is associated with arthritis development in patients with seropositive arthralgia. Ann Rheum Dis. 2011;70(1):128-33.

16. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, et al. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. Arthritis Rheum. 2000;43(1):155-63.
17. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci U S A. 2011;108(42):17372-7.
18. Juarez M, Bang H, Hammar F, Reimer U, Dyke B, Sahbudin I, et al. Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis. Ann Rheum Dis. 2016;75(6):1099-107.
19. Walsh CT, Garneau-Tsodikova S, Gatto GJ, Jr. Protein posttranslational modifications: the chemistry of proteome diversifications. Angew Chem Int Ed Engl. 2005;44(45):7342-72.
20. Darrah E, Andrade F**.** Editorial: citrullination, and carbamylation, and malondialdehyde-acetaldehyde! Oh my! Entering the forest of autoantigen modifications in rheumatoid arthritis. Arthritis Rheumatol. 2015;67(3):604-8.
21. Courties A, Gualillo O, Berenbaum F, Sellam J**.** Metabolic stress-induced joint inflammation and osteoarthritis. Osteoarthritis Cartilage. 2015;23(11):1955-65.
22. Trouw LA, Rispens T, Toes REM**.** Beyond citrullination: other post-translational protein modifications in rheumatoid arthritis. Nat Rev Rheumatol. 2017;13(6):331-9.
23. Klareskog L, Widhe M, Hermansson M, Ronnelid J**.** Antibodies to citrullinated proteins in arthritis: pathology and promise. Curr Opin Rheumatol. 2008;20(3):300-5.
24. Willemze A, Trouw LA, Toes RE, Huizinga TW**.** The influence of ACPA status and characteristics on the course of RA. Nat Rev Rheumatol. 2012;8(3):144-52.
25. van der Woude D, Syversen SW, van der Voort EI, Verpoort KN, Goll GL, van der Linden MP, et al. The ACPA isotype profile reflects long-term radiographic progression in rheumatoid arthritis. Ann Rheum Dis. 2010;69(6):1110-6.
26. Syversen SW, Gaarder PI, Goll GL, Odegard S, Haavardsholm EA, Mowinckel P, et al. High anti-cyclic citrullinated peptide levels and an algorithm of four variables predict radiographic progression in patients with rheumatoid arthritis: results from a 10-year longitudinal study. Ann Rheum Dis. 2008;67(2):212-7.
27. van der Woude D, Young A, Jayakumar K, Mertens BJ, Toes RE, van der Heijde D, et al. Prevalence of and predictive factors for sustained disease-modifying antirheumatic drug-free remission in rheumatoid arthritis: results from two large early arthritis cohorts. Arthritis Rheum. 2009;60(8):2262-71.
28. Nishimura K, Sugiyama D, Kogata Y, Tsuji G, Nakazawa T, Kawano S, et al. Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. Ann Intern Med. 2007;146(11):797-808.
29. Shi J, van Veelen PA, Mahler M, Janssen GM, Drijfhout JW, Huizinga TW, et al. Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies. Autoimmun Rev. 2014;13(3):225-30.
30. Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Horkko S, Barnard J, et al. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. Nat Med. 2007;13(10):1176-84.

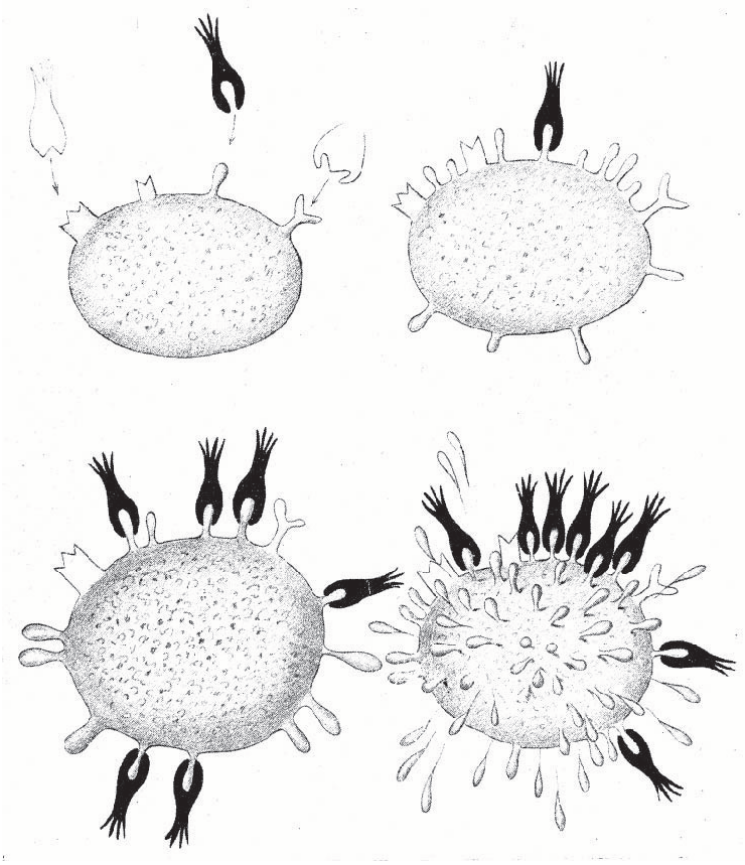
31. Holzer M, Gauster M, Pfeifer T, Wadsack C, Fauler G, Stiegler P, et al. Protein carbamylation renders high-density lipoprotein dysfunctional. Antioxid Redox Signal. 2011;14(12):2337-46.
32. Roberts JM, Veres PR, Cochran AK, Warneke C, Burling IR, Yokelson RJ, et al. Isocyanic acid in the atmosphere and its possible link to smoke-related health effects. Proc Natl Acad Sci U S A. 2011;108(22):8966-71.
33. Kalim S, Karumanchi SA, Thadhani RI, Berg AH**.** Protein carbamylation in kidney disease: pathogenesis and clinical implications. Am J Kidney Dis. 2014;64(5):793-803.
34. Scinocca M, Bell DA, Racape M, Joseph R, Shaw G, McCormick JK, et al. Antihomocitrullinated fibrinogen antibodies are specific to rheumatoid arthritis and frequently bind citrullinated proteins/peptides. J Rheumatol. 2014;41(2):270-9.
35. Reed E, Jiang X, Kharlamova N, Ytterberg AJ, Catrina AI, Israelsson L, et al. Antibodies to carbamylated alpha-enolase epitopes in rheumatoid arthritis also bind citrullinated epitopes and are largely indistinct from anti-citrullinated protein antibodies. Arthritis Res Ther. 2016;18(1):96.
36. Turunen S, Hannonen P, Koivula MK, Risteli L, Risteli J**.** Separate and overlapping specificities in rheumatoid arthritis antibodies binding to citrulline- and homocitrulline-containing peptides related to type I and II collagen telopeptides. Arthritis Res Ther. 2015;17:2.
37. Mydel P, Wang Z, Brisslert M, Hellvard A, Dahlberg LE, Hazen SL, et al. Carbamylation-dependent activation of T cells: a novel mechanism in the pathogenesis of autoimmune arthritis. J Immunol. 2010;184(12):6882-90.
38. Jiang X, Trouw LA, van Wesemael TJ, Shi J, Bengtsson C, Kallberg H, et al. Anti-CarP antibodies in two large cohorts of patients with rheumatoid arthritis and their relationship to genetic risk factors, cigarette smoking and other autoantibodies. Ann Rheum Dis. 2014;73(10):1761-8.
39. Brink M, Verheul MK, Ronnelid J, Berglin E, Holmdahl R, Toes RE, et al. Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage. Arthritis Res Ther. 2015;17:25.
40. Shi J, van de Stadt LA, Levarht EW, Huizinga TW, Hamann D, van Schaardenburg D, et al. Anti-carbamylated protein (anti-CarP) antibodies precede the onset of rheumatoid arthritis. Ann Rheum Dis. 2014;73(4):780-3.
41. Gan RW, Trouw LA, Shi J, Toes RE, Huizinga TW, Demoruelle MK, et al. Anti-carbamylated protein antibodies are present prior to rheumatoid arthritis and are associated with its future diagnosis. J Rheumatol. 2015;42(4):572-9.
42. Shi J, van Steenbergen HW, van Nies JA, Levarht EW, Huizinga TW, van der Helm-van Mil AH, et al. The specificity of anti-carbamylated protein antibodies for rheumatoid arthritis in a setting of early arthritis. Arthritis Res Ther. 2015;17:339.
43. Lac P, Racape M, Barra L, Bell DA, Cairns E**.** Relatedness of Antibodies to Peptides Containing Homocitrulline or Citrulline in Patients with Rheumatoid Arthritis. J Rheumatol. 2018;45(3):302-9.
44. Stoop JN, Liu BS, Shi J, Jansen DT, Hegen M, Huizinga TW, et al. Antibodies specific for carbamylated proteins precede the onset of clinical symptoms in mice with collagen induced arthritis. PLoS One. 2014;9(7):e102163.

45. Steinbrecher UP, Fisher M, Witztum JL, Curtiss LK. Immunogenicity of homologous low density lipoprotein after methylation, ethylation, acetylation, or carbamylation: generation of antibodies specific for derivatized lysine. J Lipid Res. 1984;25(10):1109-16.
46. Stoop JN, Fischer A, Hayer S, Hegen M, Huizinga TW, Steiner G, et al. Anticarbamylated protein antibodies can be detected in animal models of arthritis that require active involvement of the adaptive immune system. Ann Rheum Dis. 2015;74(5):949-50.
47. Guan KL, Yu W, Lin Y, Xiong Y, Zhao S. Generation of acetyllysine antibodies and affinity enrichment of acetylated peptides. Nat Protoc. 2010;5(9):1583-95.
48. Phillips DM. The presence of acetyl groups of histones. Biochem J. 1963;87:258-63.
49. Grunstein M. Histone acetylation in chromatin structure and transcription. Nature. 1997;389(6649):349-52.
50. Paolinelli R, Mendoza-Maldonado R, Cereseto A, Giacca M. Acetylation by GCN5 regulates CDC6 phosphorylation in the S phase of the cell cycle. Nat Struct Mol Biol. 2009;16(4):412-20.
51. Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell. 1997;90(4):595-606.
52. Hu LI, Lima BP, Wolfe AJ. Bacterial protein acetylation: the dawning of a new age. Mol Microbiol. 2010;77(1):15-21.
53. Butler CA, Veith PD, Nieto MF, Dashper SG, Reynolds EC. Lysine acetylation is a common post-translational modification of key metabolic pathway enzymes of the anaerobe Porphyromonas gingivalis. J Proteomics. 2015;128:352-64.
54. Konig MF, Abusleme L, Reinholdt J, Palmer RJ, Teles RP, Sampson K, et al. Aggregatibacter actinomycetemcomitans-induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis. Sci Transl Med. 2016;8(369):369ra176.
55. Mikuls TR, Payne JB, Yu F, Thiele GM, Reynolds RJ, Cannon GW, et al. Periodontitis and Porphyromonas gingivalis in patients with rheumatoid arthritis. Arthritis Rheumatol. 2014;66(5):1090-100.
56. Bright R, Thiele GM, Manavis J, Mikuls TR, Payne JB, Bartold PM. Gingival tissue, an extrasynovial source of malondialdehyde-acetaldehyde adducts, citrullinated and carbamylated proteins. J Periodontal Res. 2018;53(1):139-43.
57. Thiele GM, Duryee MJ, Anderson DR, Klassen LW, Mohring SM, Young KA, et al. Malondialdehyde-acetaldehyde adducts and anti-malondialdehyde-acetaldehyde antibodies in rheumatoid arthritis. Arthritis Rheumatol. 2015;67(3):645-55.
58. Lundberg K, Nijenhuis S, Vossenaar ER, Palmblad K, van Venrooij WJ, Klareskog L, et al. Citrullinated proteins have increased immunogenicity and arthritogenicity and their presence in arthritic joints correlates with disease severity. Arthritis Res Ther. 2005;7(3):R458-67.
59. Hill JA, Bell DA, Brintnell W, Yue D, Wehrli B, Jevnikar AM, et al. Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. J Exp Med. 2008;205(4):967-79.
60. Thiele GM, Tuma DJ, Willis MS, Miller JA, McDonald TL, Sorrell MF, et al. Soluble proteins modified with acetaldehyde and malondialdehyde are immunogenic in the absence of adjuvant. Alcohol Clin Exp Res. 1998;22(8):1731-9.

61. Lac P, Saunders S, Tutunea-Fatan E, Barra L, Bell DA, Cairns E**.** Immune responses to peptides containing homocitrulline or citrulline in the DR4-transgenic mouse model of rheumatoid arthritis. J Autoimmun. 2018;89:75-81.
62. Foy TM, Laman JD, Ledbetter JA, Aruffo A, Claassen E, Noelle RJ**.** gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. J Exp Med. 1994;180(1):157-63.
63. Bingham CO, 3rd, Moni M**.** Periodontal disease and rheumatoid arthritis: the evidence accumulates for complex pathobiologic interactions. Curr Opin Rheumatol. 2013;25(3):345-53.

PART I

ROLE OF PROTEIN MODIFICATIONS ON AUTOIMMUNITY



Adapted from Croonian Lecture "On Immunity with Special Reference to Cell Life"
Paul Erlich read 22 March 1900

Proceedings of the Royal Society, January 1899, London

Chapter 2

The role of anti-citrullinated protein antibodies in the early stages of rheumatoid arthritis

Current Opinion in Rheumatology 2016 May;(3):275-81.

Jacqueline Dekkers, René E.M. Toes, Tom W.J. Huizinga and Diane van der Woude

Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands.

Introduction

The identification of anti-citrullinated protein antibodies (ACPA) has had a major impact on the understanding of rheumatoid arthritis (RA). In the late 1990's it was described for the first time that RA patients produce autoantibodies which target peptides and proteins containing citrulline, a modified form of the amino acid arginine (1) (2). Citrullination is a posttranslational modification of protein-bound arginine into citrulline residues which is mediated by peptidyl arginine deiminase (PAD) enzymes and is essential for the generation of antigens recognized by ACPA (3). Although the physiological role of citrullination is not precisely known, it is clear that this protein modification can occur during a variety of biological processes, including inflammation. Following the identification of citrullinated proteins, several diagnostic tests were developed based on cyclic citrullinated peptides (CCP) as a test substrate for detecting ACPA. Using the CCP-assay, a highly reliable diagnostic tool became available for routine testing of antibodies directed against citrullinated epitopes in early RA patients.

The presence of ACPA in the sera of patients represents an important early biomarker and has been added to the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria for RA (4). The selected parameters in the 2010 classification criteria were designed to include early markers of disease rather than established clinical features as was the case in the 1987 ACR guidelines. The ACPA serology enables the clinician to identify recent-onset RA patients earlier, which is crucial for achieving timely control of disease progression. Using the ACPA test it is possible to distinguish two subclasses RA patients: ACPA-positive and ACPA-negative. When comparing these two subclasses of RA, major differences have been observed regarding genetic- and environmental risk factors, progression, remission, and response to treatment. In this review, we will provide an update on the latest findings concerning the ACPA maturation profile, the association between RA and the HLA DR-locus, and the hypotheses about disease pathogenesis that contribute to a greater understanding of the role of ACPA in early RA.

Auto-immunity related to RA is present long before onset of clinical symptoms

Autoantibodies are an important hallmark of RA and several classes of autoantibodies have been described that precede the development of RA, including ACPA, rheumatoid factor (RF) and the recently identified anti-carbamylated protein antibodies (5-8). Especially ACPA are of particular interest as these autoantibodies are highly specific for RA and can be found in about 50% of early RA patients. The fact that ACPA are quite rare in healthy individuals, suggests that auto-antibody positive healthy individuals are at an increased risk of developing RA (9). These findings suggest that ACPA and/or the underlying B-/T-cell responses play a prominent role in disease pathogenesis. Shortly before clinical onset of

disease, there appears to be maturation of the ACPA response which is characterized by an increase in ACPA titre, isotype switching, an increased antigen-recognition profile, and a change in Fc glycosylation pattern (10-15). Different observations strongly suggest that the development of ACPA-positive RA is based on a two-hit model. Environmental triggers and epigenetic stochastic events are thought to play a role in the initial break of tolerance leading to the formation of ACPA. A 'second hit', such as an infection or other factors, triggers the expansion of the ACPA response, which occurs relatively short before disease manifestation (16). Epidemiological studies have indicated that the HLA molecules do not play a considerable role during the first hit, but mainly contribute to the second hit that enables the expansion of the ACPA response.

ACPA can recognize a variety of citrullinated antigens, including type II collagen, fibrinogen, vimentin and many other citrullinated proteins. An increase or shift of the antigen recognition profile, epitope spreading, can be a sign of maturation of the antibody response and disease progression. Epitope spreading is a hallmark of maturation of the ACPA response and is predictive for disease progression to early RA. After disease onset, the increased citrullinated epitope-recognition profile stabilizes and does not change anymore (10). A recent 2-year follow up study enrolling 316 early RA patients in a Swedish pharmacotherapy trial suggested that disappearance of particular ACPA reactivities may be associated with a good treatment response in early RA (17). These results differ from previous reports in which the ACPA fine specificity did not seem to correlate with disease activity, progression, or response to therapy (18-20). In the case of the response against recall antigens, antibodies undergo class switching, somatic hypermutation and affinity maturation to improve the immune reaction against the antigen.

The variable region of ACPA has undergone extensive somatic hypermutation, indicative of a T-cell-dependent B-cell response (21). The avidity maturation of ACPA however, appears to be different from recall antigens. As compared with antibodies against recall antigens, ACPA display a considerably lower avidity and the ACPA response shows only limited avidity maturation over time (22, 23). The presence of these low-avidity ACPA in RA patients is associated with a higher rate of joint destruction. ACPA can activate the complement system and can therefore play a role in the complement-mediated recruitment of inflammatory cells (24), which suggests that ACPA could be directly involved in the disease process. Moreover, ACPA-immune complexes combined with IgM or IgA RF can directly trigger Fcγ receptors on macrophages and mast cells leading to the production of proinflammatory cytokines which contribute to RA synovitis (25, 26).

Maturation of antibody responses leads to a shift in isotype which enables the activation of other immune effector mechanisms. ACPA can use multiple isotypes, and these ACPA isotypes are already present before onset of RA (27). In addition, the number of different ACPA isotypes is predictive for the development of radiological damage (28). Similar to the epitope-recognition profile, the ACPA isotype profile appears not to expand anymore during

disease progression, indicating that maturation of the ACPA response takes place before onset of arthritis. As mentioned above, the fragment crystallisable (Fc) region of an antibody interacts with Fc receptors of immune effector cells and the complement system, and thus determines which immune effector mechanisms can be recruited by the antibody. The glycosylation of the IgG-Fc region of ACPA has been reported to be different from non-ACPA IgG. The Fc region of ACPA-IgG_1 contains reduced numbers of sialic acid and galactose residues (29), a feature which is generally considered to render IgG antibodies proinflammatory (30). The changes in ACPA Fc glycosylation pattern become more prominent around 3 months before onset of RA (15). Differences in glycosylation pattern between Ig isotypes might influence their affinity for Fc receptors. A recent study showed that ACPA-IgG_1 has a different Fc glycan profile compared to non-CCP2 reactive IgG_1 (30), a particularity which can influence the affinity of ACPA IgG to Fc receptors and complement and may modulate ACPA effector- and immune-regulatory functions (31). In conclusion, all these different autoantibody characteristics evolve and mature before disease onset, and once patients present with arthritis, the ACPA response is generally increased in titre, uses more isotypes, displays a different glycosylation pattern, and are cross-reactive towards different citrullinated proteins (Figure 1).

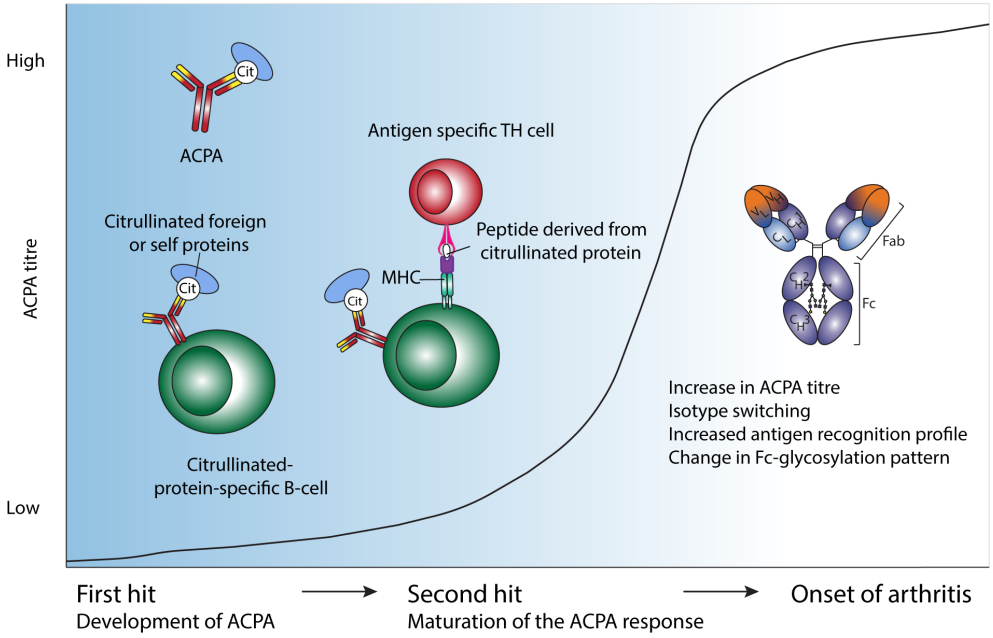


Figure 1. Maturation of the ACPA-response. Antibodies reactive towards citrullinated proteins are already present in the preclinical phase of RA. Environmental triggers and epigenetic stochastic events are thought to play a role in the 'first hit', leading to the formation of ACPA. A 'second hit', such as an infection, triggers further expansion and maturation of the ACPA response. Once the disease manifests itself, the ACPA response is generally increased in titre, uses more isotypes, has a different glycosylation pattern, and an increased antigen recognition profile towards various citrullinated proteins. Abbreviations: MHC, major histocompatibility complex; T_H, T-helper cell; Fab, fragment antigen-binding; Fc, fragment crystallisable.

HLA class II associations in rheumatoid arthritis

The most important genetic risk factor for ACPA positive RA is the HLA class II region. RA, like many other autoimmune diseases, is characterized by a strong association with variants in the human leucocyte antigen (HLA) class II region. These HLA-associations differ in ACPA-positive and ACPA-negative disease (32-35), highlighting the complexity of pathogenic mechanisms underlying HLA associations in RA. The HLA class II region encodes for HLA-DR, HLA-DQ and HLA-DP proteins and is involved in antigen presentation to HLA-class II restricted $CD4^+$ T-helper cells. In 1976, analysis of mixed lymphocyte cultures from RA patients revealed that these individuals had certain HLA-DR4 molecules in common (36). The HLA haplotypes that encode for the HLA-DR4 molecules were found to be characterized by the so-called 'HLA-shared epitope (SE)', a common amino acid sequence in the HLA-DRB1

chain (37). Recent genome wide analysis revealed specific amino acids at positions 11, 13, 71 and 74 of the HLA-DRB1 chain as well was single-amino-acid polymorphisms at position nine of HLA-B and HLA-DPB1 are associated with the greatest risk for RA (38). Amino acid positions 11 and 13 of HLA-DRB1 are among the most polymorphic and are highly relevant for the shaping of peptide binding pockets located in peptide-binding groove of the HLA molecule. It is therefore not surprisingly that the statistically significant amino acid positions are those involved in peptide presentation.

The HLA SE alleles, are now known to be specifically associated with ACPA-positive RA (38) (32). Conversely, HLA-DRB1*13 alleles haplotypes have been found to protect against the development of ACPA-positive RA (33, 34). A possible explanation for the association of the 'HLA-shared epitope' with ACPA-positive RA might be that peptide presentation by the 'HLA-shared epitope' HLA molecules can facilitate the activation of CD4+ T-cells which provide help to ACPA-producing B-cells. In ACPA positive RA, ACPA are cross-reactive and bind to a wide variety of citrullinated self-proteins which indicates to a loss of B-cell tolerance. However, it is unclear to what extent T-cell tolerance is lost. Identification of citrullinated epitopes recognized by autoreactive T-cells in patients with RA has proven difficult. Analysis using peptide-HLA tetramers and *in vitro* T-cell responses to candidate epitopes revealed T-cell recognition of several citrullinated epitopes in humans (39-42).

The HLA-DRB1*13 alleles which are protective for RA, carry a five amino-acid sequence called: DERAA. The DERAA sequence is also expressed by many microbes and in a self-protein vinculin. Citrullinated vinculin is expressed in the inflamed synovial membrane and was recently identified as a novel autoantigen for ACPA antibodies (43). It is proposed that molecular mimicry of self-proteins with pathogenic microbial proteins might lead to a break of T-cell tolerance. Indeed, it was recently shown, that T-cells present in HLA-DRB1*13-negative donors were able to specifically recognize a DERAA-containing vinculin epitope that cross-react with DERAA sequences derived from pathogens (44). However, many T-cell responses are absent in HLA-DR13+ donors, indicating the induction of DERAA-specific T-cell tolerance in these donors. Together, these studies suggests that the HLA class II locus can directly influence the maturation of the ACPA response via antigen-specific T-cells, providing help to ACPA-producing B-cells and enabling the maturation of the citrullinated protein-specific autoantibody response.

Pathogenic role of the immune response against citrullinated proteins

Besides the diagnostic application of ACPA as a biomarker, several clinical observations suggest that ACPA could play a direct role in disease pathology. First, ACPA may be found early in the course of disease, up to 7 years before RA manifests (5, 6). Second, various follow-up studies revealed that ACPA-positive patients with recent-onset RA develop more bone erosions compared to ACPA-negative RA patients (45-48). Third, bone loss and reduced bone mineral density can be found in healthy ACPA-positive individuals, even before clinical onset of arthritis (49). Selective B-cell depletion using rituximab has been found to be effective in the treatment of RA (50-52), providing evidence for the involvement of B-cells in the pathogenesis of RA. ACPA-producing B-cells are found to be enriched in synovial fluid (21, 53), which suggests that ACPA can be produced locally and directly contribute to synovial inflammation. Moreover, the numbers of ACPA-producing B-cells in the blood of RA patients correlate with ACPA serum levels (54).

Functional studies showed that immune complexes formed with ACPA mediate effector functions via Fc-γ receptors (55), and can induce complement activation (24) and enhanced neutrophil extracellular trap formation (56). In addition, there are reports that purified ACPA can induce osteoclastogenesis and bone resorption in mice (57), suggesting a direct link between ACPA and more severe joint destruction. So far, only two experimental studies succeeded in showing *in vivo* that ACPA may facilitate the transition from autoimmunity to inflammation. Transfer of antibodies specific to citrullinated fibrinogen (58) and transfer of antibodies targeting citrullinated-collagen (59) to mice with mild experimental arthritis led to disease exacerbation. It is interesting that no other positive data have been reported allowing that these two positive papers are similar as the many non-replicated preclinical papers in other fields such as oncology (60).

RA patients receiving Abatacept show reduced levels of ACPA and RF in response to treatment (61). Moreover, a Swedish pharmacotherapy trial reported a decline of all ACPA levels independent of the clinical response on disease activity during the first three months of methotrexate treatment (17). However, effective treatment of established arthritis does not necessarily lead to reduced ACPA levels or a change in ACPA composition. For example, a Canadian cohort in early arthritis patients found that anti-CCP antibody fluctuations did not relate to clinical scores such as disease activity scores and the presence of erosions (62). These findings suggest that autoantibody producing B-cells rather than the autoantibodies produced may be responsible for disease pathogenesis. Activated B-cells secrete pro-inflammatory cytokines, such as IL-6 and TNF, and ACPA-producing B-cells are found to be increased in the inflamed synovial membrane (16).

Besides ACPA, other autoantibodies and/or auto-antibody-producing B-cells may also be involved in RA pathogenesis. Similar to ACPA, both rheumatoid factor and anti-CarP antibodies are associated with disease severity and persistence (8, 63, 64). Anti-CarP

autoantibodies recognize carbamylated proteins containing a homocitrulline, a post-translational modification of lysine driven by cyanate, and can be found in patients with ACPA-negative RA. More recently, antibodies targeting another post-translational modification, malondialdehyde/acetaldehyde (MAA) adducts, are found to be increased in RA patients and this antibody response is associated with the presence of ACPA (65). Similar to carbamylation, MAA adducts are capable of the modification of a lysine. These findings raise the question why RA patients produce auto-antibodies towards post-translational modified proteins and whether these autoantibodies are implicated in disease pathogenesis. Further research is needed to confirm the current observations of anti-CarP antibodies in ACPA-negative and positive RA patients, and to determine whether these biomarkers can provide additional value next to the CCP2 test.

Conclusion

ACPA have proven to be a very useful biomarker for diagnosing RA and for predicting a severe disease course. Future investigations on the role of ACPA, other autoantibodies, and ACPA-producing B-cells in RA may provide further insight in and understanding of the underlying disease pathogenesis. Follow-up studies of RA patients may provide useful information on the fluctuation of ACPA levels and changes in ACPA composition during disease progression and treatment. Together, these observations may allow for new approaches to treat RA at an early, preclinical, stage of disease, and thus enable prevention of the transition of autoimmunity to inflammation and autoimmune disease.

References

1. Vincent C, Serre G, Fournie B, Fournie A, Soleilhavoup JP**.** Natural IgG to epidermal cytokeratins vs IgG to the stratum corneum of the rat oesophagus epithelium, so-called 'antikeratin antibodies', in rheumatoid arthritis and other rheumatic diseases. J Autoimmun. 1991;4(3):493-505.
2. Schellekens GA, de Jong BA, van den Hoogen FH, van de Putte LB, van Venrooij WJ**.** Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. J Clin Invest. 1998;101(1):273-81.
3. Vossenaar ER, Zendman AJ, van Venrooij WJ, Pruijn GJ**.** PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. Bioessays. 2003;25(11):1106-18.
4. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis. 2010;69(9):1580-8.
5. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum. 2003;48(10):2741-9.
6. Nielen MM, van Schaardenburg D, Reesink HW, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MH, et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum. 2004;50(2):380-6.
7. Berglin E, Johansson T, Sundin U, Jidell E, Wadell G, Hallmans G, et al. Radiological outcome in rheumatoid arthritis is predicted by presence of antibodies against cyclic citrullinated peptide before and at disease onset, and by IgA-RF at disease onset. Ann Rheum Dis. 2006;65(4):453-8.
8. Shi J, van de Stadt LA, Levarht EW, Huizinga TW, Hamann D, van Schaardenburg D, et al. Anti-carbamylated protein (anti-CarP) antibodies precede the onset of rheumatoid arthritis. Ann Rheum Dis. 2014;73(4):780-3.
9. Terao C, Ohmura K, Ikari K, Kawaguchi T, Takahashi M, Setoh K, et al. Effects of smoking and shared epitope on the production of anti-citrullinated peptide antibody in a Japanese adult population. Arthritis Care Res (Hoboken). 2014;66(12):1818-27.
10. van der Woude D, Rantapaa-Dahlqvist S, Ioan-Facsinay A, Onnekink C, Schwarte CM, Verpoort KN, et al. Epitope spreading of the anti-citrullinated protein antibody response occurs before disease onset and is associated with the disease course of early arthritis. Ann Rheum Dis. 2010;69(8):1554-61.
11. Ioan-Facsinay A, Willemze A, Robinson DB, Peschken CA, Markland J, van der Woude D, et al. Marked differences in fine specificity and isotype usage of the anti-citrullinated protein antibody in health and disease. Arthritis Rheum. 2008;58(10):3000-8.
12. van de Stadt LA, de Koning MH, van de Stadt RJ, Wolbink G, Dijkmans BA, Hamann D, et al. Development of the anti-citrullinated protein antibody repertoire prior to the onset of rheumatoid arthritis. Arthritis Rheum. 2011;63(11):3226-33.
13. van de Stadt LA, van der Horst AR, de Koning MH, Bos WH, Wolbink GJ, van de Stadt RJ, et al. The extent of the anti-citrullinated protein antibody repertoire is associated with arthritis development in patients with seropositive arthralgia. Ann Rheum Dis. 2011;70(1):128-33.

14. Brink M, Hansson M, Mathsson L, Jakobsson PJ, Holmdahl R, Hallmans G, et al. Multiplex analyses of antibodies against citrullinated peptides in individuals prior to development of rheumatoid arthritis. Arthritis Rheum. 2013;65(4):899-910.
15. Rombouts Y, Ewing E, van de Stadt LA, Selman MH, Trouw LA, Deelder AM, et al. Anti-citrullinated protein antibodies acquire a pro-inflammatory Fc glycosylation phenotype prior to the onset of rheumatoid arthritis. Ann Rheum Dis. 2015;74(1):234-41.
16. Koning F, Thomas R, Rossjohn J, Toes RE**.** Coeliac disease and rheumatoid arthritis: similar mechanisms, different antigens. Nat Rev Rheumatol. 2015;11(8):450-61.
17. Kastbom A, Forslind K, Ernestam S, Geborek P, Karlsson JA, Petersson IF, et al. Changes in the anticitrullinated peptide antibody response in relation to therapeutic outcome in early rheumatoid arthritis: results from the SWEFOT trial. Ann Rheum Dis. 2014.
18. van Beers JJ, Willemze A, Jansen JJ, Engbers GH, Salden M, Raats J, et al. ACPA fine-specificity profiles in early rheumatoid arthritis patients do not correlate with clinical features at baseline or with disease progression. Arthritis Res Ther. 2013;15(5):R140.
19. Scherer HU, van der Woude D, Willemze A, Trouw LA, Knevel R, Syversen SW, et al. Distinct ACPA fine specificities, formed under the influence of HLA shared epitope alleles, have no effect on radiographic joint damage in rheumatoid arthritis. Ann Rheum Dis. 2011;70(8):1461-4.
20. Fisher BA, Plant D, Brode M, van Vollenhoven RF, Mathsson L, Symmons D, et al. Antibodies to citrullinated alpha-enolase peptide 1 and clinical and radiological outcomes in rheumatoid arthritis. Ann Rheum Dis. 2011;70(6):1095-8.
21. Amara K, Steen J, Murray F, Morbach H, Fernandez-Rodriguez BM, Joshua V, et al. Monoclonal IgG antibodies generated from joint-derived B cells of RA patients have a strong bias toward citrullinated autoantigen recognition. J Exp Med. 2013;210(3):445-55.
22. Suwannalai P, Scherer HU, van der Woude D, Ioan-Facsinay A, Jol-van der Zijde CM, van Tol MJ, et al. Anti-citrullinated protein antibodies have a low avidity compared with antibodies against recall antigens. Ann Rheum Dis. 2011;70(2):373-9.
23. Suwannalai P, van de Stadt LA, Radner H, Steiner G, El-Gabalawy HS, Zijde CM, et al. Avidity maturation of anti-citrullinated protein antibodies in rheumatoid arthritis. Arthritis Rheum. 2012;64(5):1323-8.
24. Trouw LA, Haisma EM, Levarht EWN, van der Woude D, Ioan-Facsinay A, Daha VR, et al. Anti-Cyclic Citrullinated Peptide Antibodies From Rheumatoid Arthritis Patients Activate Complement via Both the Classical and Alternative Pathways. Arthritis and Rheumatism. 2009;60(7):1923-31.
25. Anquetil F, Clavel C, Offer G, Serre G, Sebbag M**.** IgM and IgA rheumatoid factors purified from rheumatoid arthritis sera boost the Fc receptor- and complement-dependent effector functions of the disease-specific anti-citrullinated protein autoantibodies. J Immunol. 2015;194(8):3664-74.
26. Suurmond J, Rivellese F, Dorjee AL, Bakker AM, Rombouts YJ, Rispens T, et al. Toll-like receptor triggering augments activation of human mast cells by anti-citrullinated protein antibodies. Ann Rheum Dis. 2015;74(10):1915-23.

27. Kokkonen H, Mullazehi M, Berglin E, Hallmans G, Wadell G, Ronnelid J, et al. Antibodies of IgG, IgA and IgM isotypes against cyclic citrullinated peptide precede the development of rheumatoid arthritis. Arthritis Res Ther. 2011;13(1):R13.
28. van der Woude D, Syversen SW, van der Voort EI, Verpoort KN, Goll GL, van der Linden MP, et al. The ACPA isotype profile reflects long-term radiographic progression in rheumatoid arthritis. Ann Rheum Dis. 2010;69(6):1110-6.
29. Scherer HU, van der Woude D, Ioan-Facsinay A, el Bannoudi H, Trouw LA, Wang J, et al. Glycan profiling of anti-citrullinated protein antibodies isolated from human serum and synovial fluid. Arthritis Rheum. 2010;62(6):1620-9.
30. Lundstrom SL, Fernandes-Cerqueira C, Ytterberg AJ, Ossipova E, Hensvold AH, Jakobsson PJ, et al. IgG Antibodies to Cyclic Citrullinated Peptides Exhibit Profiles Specific in Terms of IgG Subclasses, Fc-Glycans and a Fab-Peptide Sequence. Plos One. 2014;9(11).
31. Karsten CM, Kohl J**.** The immunoglobulin, IgG Fc receptor and complement triangle in autoimmune diseases. Immunobiology. 2012;217(11):1067-79.
32. Huizinga TW, Amos CI, van der Helm-van Mil AH, Chen W, van Gaalen FA, Jawaheer D, et al. Refining the complex rheumatoid arthritis phenotype based on specificity of the HLA-DRB1 shared epitope for antibodies to citrullinated proteins. Arthritis Rheum. 2005;52(11):3433-8.
33. Oka S, Furukawa H, Kawasaki A, Shimada K, Sugii S, Hashimoto A, et al. Protective effect of the HLA-DRB1*13:02 allele in Japanese rheumatoid arthritis patients. PLoS One. 2014;9(6):e99453.
34. van der Woude D, Lie BA, Lundstrom E, Balsa A, Feitsma AL, Houwing-Duistermaat JJ, et al. Protection against anti-citrullinated protein antibody-positive rheumatoid arthritis is predominantly associated with HLA-DRB1*1301: a meta-analysis of HLA-DRB1 associations with anti-citrullinated protein antibody-positive and anti-citrullinated protein antibody-negative rheumatoid arthritis in four European populations. Arthritis Rheum. 2010;62(5):1236-45.
35. Han B, Diogo D, Eyre S, Kallberg H, Zhernakova A, Bowes J, et al. Fine mapping seronegative and seropositive rheumatoid arthritis to shared and distinct HLA alleles by adjusting for the effects of heterogeneity. Am J Hum Genet. 2014;94(4):522-32.
36. Stastny P**.** Mixed lymphocyte cultures in rheumatoid arthritis. J Clin Invest. 1976;57(5):1148-57.
37. Gregersen PK, Silver J, Winchester RJ**.** The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum. 1987;30(11):1205-13.
38. Raychaudhuri S, Sandor C, Stahl EA, Freudenberg J, Lee HS, Jia X, et al. Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis. Nat Genet. 2012;44(3):291-6.
39. Scally SW, Petersen J, Law SC, Dudek NL, Nel HJ, Loh KL, et al. A molecular basis for the association of the HLA-DRB1 locus, citrullination, and rheumatoid arthritis. Journal of Experimental Medicine. 2013;210(12):2569-82.

40. Feitsma AL, van der Voort EIH, Franken KLMC, el Bannoudi H, Elferink BG, Drijfhout JW, et al. Identification of Citrullinated Vimentin Peptides as T Cell Epitopes in HLA-DR4-Positive Patients With Rheumatoid Arthritis. Arthritis and Rheumatism. 2010;62(1):117-25.
41. James EA, Rieck M, Pieper J, Gebe JA, Yue BB, Tatum M, et al. Citrulline-Specific Th1 Cells Are Increased in Rheumatoid Arthritis and Their Frequency Is Influenced by Disease Duration and Therapy. Arthritis & Rheumatology. 2014;66(7):1712-22.
42. Law SC, Street S, Yu CHA, Capini C, Ramnoruth S, Nel HJ, et al. T-cell autoreactivity to citrullinated autoantigenic peptides in rheumatoid arthritis patients carrying HLA-DRB1 shared epitope alleles. Arthritis Research & Therapy. 2012;14(3).
43. van Beers JJ, Schwarte CM, Stammen-Vogelzangs J, Oosterink E, Bozic B, Pruijn GJ**.** The rheumatoid arthritis synovial fluid citrullinome reveals novel citrullinated epitopes in apolipoprotein E, myeloid nuclear differentiation antigen, and beta-actin. Arthritis Rheum. 2013;65(1):69-80.
44. van Heemst J, Jansen DTSL, Polydorides S, Moustakas AK, Bax M, Feitsma AL, et al. Crossreactivity to vinculin and microbes provides a molecular basis for HLA-based protection against rheumatoid arthritis. Nature Communications. 2015;6.
45. Courvoisier N, Dougados M, Cantagrel A, Goupille P, Meyer O, Sibilia J, et al. Prognostic factors of 10-year radiographic outcome in early rheumatoid arthritis: a prospective study. Arthritis Res Ther. 2008;10(5):R106.
46. Syversen SW, Goll GL, van der Heijde D, Landewe R, Lie BA, Odegard S, et al. Prediction of radiographic progression in rheumatoid arthritis and the role of antibodies against mutated citrullinated vimentin: results from a 10-year prospective study. Ann Rheum Dis. 2010;69(2):345-51.
47. Plant D, Thomson W, Lunt M, Flynn E, Martin P, Eyre S, et al. The role of rheumatoid arthritis genetic susceptibility markers in the prediction of erosive disease in patients with early inflammatory polyarthritis: results from the Norfolk Arthritis Register. Rheumatology (Oxford). 2011;50(1):78-84.
48. Jilani AA, Mackworth-Young CG**.** The role of citrullinated protein antibodies in predicting erosive disease in rheumatoid arthritis: a systematic literature review and meta-analysis. Int J Rheumatol. 2015;2015:728610.
49. Kleyer A, Finzel S, Rech J, Manger B, Krieter M, Faustini F, et al. Bone loss before the clinical onset of rheumatoid arthritis in subjects with anticitrullinated protein antibodies. Ann Rheum Dis. 2014;73(5):854-60.
50. Cambridge G, Leandro MJ, Edwards JC, Ehrenstein MR, Salden M, Bodman-Smith M, et al. Serologic changes following B lymphocyte depletion therapy for rheumatoid arthritis. Arthritis Rheum. 2003;48(8):2146-54.
51. Nakou M, Katsikas G, Sidiropoulos P, Bertsias G, Papadimitraki E, Raptopoulou A, et al. Rituximab therapy reduces activated B cells in both the peripheral blood and bone marrow of patients with rheumatoid arthritis: depletion of memory B cells correlates with clinical response. Arthritis Res Ther. 2009;11(4):R131.
52. Trouvin AP, Jacquot S, Grigioni S, Curis E, Dedreux I, Roucheux A, et al. Usefulness of monitoring of B cell depletion in rituximab-treated rheumatoid arthritis patients in order to

predict clinical relapse: a prospective observational study. Clin Exp Immunol. 2015;180(1):11-8.
53. Snir O, Widhe M, Hermansson M, von Spee C, Lindberg J, Hensen S, et al. Antibodies to several citrullinated antigens are enriched in the joints of rheumatoid arthritis patients. Arthritis Rheum. 2010;62(1):44-52.
54. Kerkman PF, Rombouts Y, van der Voort EIH, Trouw LA, Huizinga TWJ, Toes REM, et al. Circulating plasmablasts/plasmacells as a source of anticitrullinated protein antibodies in patients with rheumatoid arthritis. Annals of the Rheumatic Diseases. 2013;72(7):1259-63.
55. Laurent L, Clavel C, Lemaire O, Anquetil F, Cornillet M, Zabraniecki L, et al. Fc gamma receptor profile of monocytes and macrophages from rheumatoid arthritis patients and their response to immune complexes formed with autoantibodies to citrullinated proteins. Annals of the Rheumatic Diseases. 2011;70(6):1052-9.
56. Carmona-Rivera C, Khandpur R, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight J, et al. Neutrophil extracellular traps are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. Journal of Immunology. 2013;190.
57. Harre U, Georgess D, Bang H, Bozec A, Axmann R, Ossipova E, et al. Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. J Clin Invest. 2012;122(5):1791-802.
58. Kuhn KA, Kulik L, Tomooka B, Braschler KJ, Arend WP, Robinson WH, et al. Antibodies against citrullinated proteins enhance tissue injury in experimental autoimmune arthritis. Journal of Clinical Investigation. 2006;116(4):961-73.
59. Uysal H, Bockermann R, Nandakumar KS, Sehnert B, Bajtner E, Engstrom A, et al. Structure and pathogenicity of antibodies specific for citrullinated collagen type II in experimental arthritis. Journal of Experimental Medicine. 2009;206(2):449-62.
60. Begley CG, Ellis LM**.** Drug development: Raise standards for preclinical cancer research. Nature. 2012;483(7391):531-3.
61. Scarsi M, Paolini L, Ricotta D, Pedrini A, Piantoni S, Caimi L, et al. Abatacept Reduces Levels of Switched Memory B Cells, Autoantibodies, and Immunoglobulins in Patients with Rheumatoid Arthritis. Journal of Rheumatology. 2014;41(4):666-72.
62. Barra L, Bykerk V, Pope JE, Haraoui BP, Hitchon CA, Thome JC, et al. Anticitrullinated Protein Antibodies and Rheumatoid Factor Fluctuate in Early Inflammatory Arthritis and Do Not Predict Clinical Outcomes. Journal of Rheumatology. 2013;40(8):1259-67.
63. Humphreys J, Verheul M, Barton A, Fu B, Toes R, Symmons D, et al. Association of anti-carbamylated protein antibodies with long-term disability and increased disease activity in patients with early inflammatory arthritis: results from the Norfolk Arthritis Register. Lancet. 2015;385 Suppl 1:S44.
64. Brink M, Verheul MK, Ronnelid J, Berglin E, Holmdahl R, Toes RE, et al. Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage. Arthritis Res Ther. 2015;17:25.
65. Thiele GM, Duryee MJ, Anderson DR, Klassen LW, Mohring SM, Young KA, et al. Malondialdehyde-acetaldehyde adducts and anti-malondialdehyde-acetaldehyde antibodies in rheumatoid arthritis. Arthritis Rheumatol. 2015;67(3):645-55.

Chapter 3

Breach of autoreactive B cell tolerance by post-translationally modified proteins

Annals of the Rheumatic Diseases. 2017 Aug;76(8):1449-1457

Jacqueline S. Dekkers[1*], Marije K. Verheul[1*], Jeroen N. Stoop[1], Bisheng Liu[1], Andreea Ioan-Facsinay[1] Peter A. van Veelen[2], Arnoud H. de Ru[2], George M.C. Janssen[2], Martin Hegen[3], Steve Rapecki[4], Tom W. J. Huizinga[1], Leendert A. Trouw[1], René E.M. Toes[1]

* These authors contributed equally

1. Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands.
2. Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands.
3. Inflammation and Immunology Research Unit, Pfizer Worldwide Research and Development, USA.
4. UCB Pharma, UK

ABSTRACT

Objectives

Over 50% of rheumatoid arthritis (RA) patients harbor a variety of Anti-Modified Protein Antibodies (AMPA) against different post-translationally modified (PTM) proteins, including anti-carbamylated protein (anti-CarP) antibodies. At present it is unknown how AMPA are generated and how autoreactive B cell responses against PTM proteins are induced. Here we studied whether PTM foreign antigens can breach B cell tolerance towards PTM self-proteins.

Methods

Serum reactivity towards five carbamylated proteins was determined for 160 RA-patients and 40 healthy individuals. Antibody cross-reactivity was studied by inhibition experiments. Mass spectrometry was performed to identify carbamylated self-proteins in human rheumatic joint tissue. Mice were immunized with carbamylated- or non-modified (auto)antigens and analyzed for autoantibody responses.

Results

We show that anti-CarP antibodies in RA are highly cross-reactive towards multiple carbamylated proteins, including modified self- as well as modified non-self proteins. Studies in mice show that anti-CarP antibody responses recognizing carbamylated self-proteins are not only induced by immunization with carbamylated self-proteins but also by immunization with carbamylated proteins of non-self origin. Similar to the data observed with sera from RA patients, the murine anti-CarP antibody response was, both at the monoclonal- and polyclonal level, highly cross-reactive towards multiple carbamylated proteins, including carbamylated self-proteins.

Conclusions

Self-reactive AMPA-responses can be induced by exposure to foreign proteins containing PTM. These data show how autoreactive B cell responses against PTM self-proteins can be induced by exposure to PTM foreign proteins and provide new insights on the breach of autoreactive B cell tolerance.

Introduction

Autoimmunity in rheumatoid arthritis (RA) patients is characterized by a spectrum of anti-modified protein antibodies (AMPA) directed against post-translationally modified (PTM) proteins. The best-known AMPA in RA are autoantibodies directed against citrullinated proteins. Anti-citrullinated protein antibodies (ACPA) target proteins that have undergone a post-translational modification of arginine into citrulline by an enzymatic process mediated by peptidylarginine deiminases (PAD) (1, 2). The identification of ACPA as specific serological marker have had a major impact on the understanding of RA and disease prognosis as their presence predicts a more destructive disease process (3-7). Much less is known about the occurrence and aetiology of other AMPA responses in RA such as autoantibodies directed to malondialdehyde-acetaldehyde (MAA) adducts, acetylated antigens, and carbamylated proteins (8-11). Anti-carbamylated protein (anti-CarP) autoantibodies recognize carbamylated proteins containing a homocitrulline, a PTM structurally similar to citrulline (8, 12). Like ACPA and rheumatoid factor, also anti-CarP antibodies can be detected in serum many years before RA manifestation (13-18) and similar to these autoantibodies, the presence of anti-CarP antibodies is predictive of increased radiological damage (13). In contrast to deimination (citrullination), carbamylation occurs through an enzyme-independent reaction in which a lysine is converted into a homocitrulline through a reaction with cyanate (19), (Figure 1A).

With the presence of various AMPA responses in RA, PTM proteins have been implicated in the breach of autoreactive B cell tolerance leading to the formation of these autoantibodies (20, 21). As AMPA have undergone isotype switching and somatic hypermutation, it is often speculated that AMPA-producing B cells have received T cell help from autoreactive T cells recognizing the same PTM self-proteins. So far, attempts to provide more insight into how autoreactive B cell responses against citrullinated proteins are induced have been hampered by the fact that ACPA do not occur in murine models of arthritis (22).

Interestingly, anti-CarP antibodies, do occur in mice with collagen-induced arthritis (CIA) (23). The kinetics of anti-CarP antibodies in CIA mice display similarity to RA as these antibodies can be detected before disease onset. In addition, humoral responses to carbamylated proteins are only present in arthritis models that require active involvement of the adaptive immune system (24). Given the observation that anti-CarP antibodies, in contrast to ACPA, do occur in both humans and mice, we here investigated for the first time how PTM (foreign) proteins could contribute to a breach of B cell tolerance.

Our findings show that autoreactive B cell responses against PTM proteins can be induced by exposure to PTM foreign proteins and provide new insights on the breach of autoreactive B cell tolerance by foreign proteins.

Methods

Human serum samples

Serum samples from 160 RA patients of the Leiden Early Arthritis Cohort (EAC) (25) and 40 healthy controls were used to study anti-CarP antibody cross-reactivity. RA patients fulfilled the 1987 RA classification criteria. All subjects provided informed consent prior to inclusion and ethical permission was provided by the institutional review board.

Carbamylation and citrullination

Fetal calf serum (FCS, Bodinco), myelin basic protein (MBP, Sigma), human serum albumin (HSA, Sigma), H1 Histone (H1 Merck Millipore), Prothrombin (ProT, provided by Prof. Blom Malmö, Sweden), ovalbumin (OVA, Sigma-Aldrich) and mouse albumin (mAlb, EMD Millipore) were incubated with 1M potassium cyanate (Sigma-Aldrich) during 12 hours at 37°C, followed by dialysis. Fibrinogen, mouse (Cell Sciences) and human (Sigma-Aldrich), was incubated with 0.5M potassium cyanate during 7 days. For citrullination; 10mg FCS or 2mg fibrinogen in 1ml containing 0.1M Tris-HCl pH 7.6 and 0.15M $CaCl_2$ was incubated with 40U PAD4 (Sigma-Aldrich) for 3 hours at 37°C. Protein carbamylation and citrullination was determined afterwards by in-house developed and standardized ELISAs confirmed by mass spectrometry.

Detection of human anti-CarP antibodies

Carbamylated and non-modified proteins were coated at 10ug/ml (diluted in 0.1M carbonate-bicarbonate buffer with a pH of 9.6) and incubated overnight on Nunc Maxisorp plates (Thermo Scientific) (8, 23). Plates were blocked for at least 6 hours with PBS/1% BSA (Sigma). Sera were diluted 50x in PBS/1%BSA/0.05%Tween (PBT) and incubated overnight. As standard, serial dilutions of a pool of positive sera was used. Binding of human IgG was detected using in PBT-diluted rabbit anti-human IgG conjugated to HRP (horseradish perixodase, DAKO, P0214), which was visualized with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)). Washing with PBS/0.05% Tween was carried out between steps. All incubations, but the ABTS detection took place at 4 °C. The reactivity to the non-modified protein was subtracted from the reactivity to the corresponding carbamylated protein. The cut-off for positivity was set as mean plus two times the standard deviation of healthy individuals. For inhibition assays, sera were pre-incubated for one hour with 0 or 0.2mg/ml carbamylated or non-modified versions of one from five antigens before addition of serum samples to the ELISA plate.

Detection of mouse anti-CarP antibodies

Non-modified or modified proteins were coated overnight at a concentration of 10 µg/ml (diluted in pH 9.6 0.1 M carbonate-bicarbonate buffer) on Nunc Maxisorp plates (Thermo

Scientific). The plates were washed with PBS/0.05% Tween (Sigma) and subsequently blocked for 6 hours at 4°C with 100 µl of PBS/1% BSA (Sigma). After washing, the wells were incubated with 50 µl serum 1/50 diluted in PBS/1% BSA/0.05% Tween. The ELISA plates were incubated overnight at 4 °C. Total Ig, IgG1, and IgG2a were detected using HRP-conjugated rabbit anti-mouse Ig antibody (Dako), HRP-conjugated goat anti-mouse IgG2a, HRP-conjugated goat anti-mouse IgG1 (all from Southern Biotec). HRP enzyme activity was visualized using ABTS. As a standard, serial dilutions of a pooled serum sample from mice with CIA were used.

Sample preparation for mass spectrometric analysis

RA joint tissue samples were obtained from knee-replacement surgery leftover material (Department of Orthopedic surgery, LUMC). This procedure was approved by the local ethical committee. Synovial tissue samples (20 mg) from RA and OA patients obtained from joint replacement surgery were washed with PBS to remove adherent body fluids such as synovial fluid and blood. Samples were incubated in ST lysis buffer (4% SDS in 0.1 M Tris-Cl pH 7.6) for 15 min at 70°C. Initially, SDS lysates were subjected to FASP II as described above, but yielding low numbers of carbamylated peptide hits. In contrast, subsequent treatment of the samples with trypsin yielded many more hits. Therefore, the synovial tissue samples (after their extraction with hot SDS to remove adherent and easily soluble protein) were digested with trypsin using the following procedure; samples were incubated in 100 µl 100 mM DTT in 25 mM NH_4HCO_3 for 20 minutes at 54°C. After centrifugation, the supernatant was saved and the pellet incubated in 150 µl 15 mM iodoacetamide in 25 mM NH_4HCO_3 for 30 minutes at room temperature. After centrifugation, the supernatant was saved and the pellet incubated in 200 µl 25 mM NH_4HCO_3 containing 10 µg trypsin for 4 hours at 37°C. The combined supernatants from DTT and iodoacetamide incubation were concentrated on a 30 kDa filter (Microcon, Millipore), washed 3 times with 100 µl 25 mM NH_4HCO_3 and also incubated with 1 µg trypsin for 4 hours at 37°C. Next, the supernatant containing digested protein from the pellet was added to the digest on the filter. The filter was washed once with 100 µl 0.5 M NaCl. Peptides were recovered from the filtrate and subjected to solid phase extraction on C18 cartridges (Oasis HLB Waters).

Proteome analysis and mass spectrometric identification of carbamylation

Peptides were analyzed via on-line C18-nano-HPLC-MS with a system consisting of an Easy nLC 1000 gradient HPLC system (Thermo Scientific), and a Q-Exactive mass spectrometer (Thermo Scientific). Fractions were injected onto a homemade precolumn (100 µm × 15 mm; Reprosil-Pur C18-AQ 3 µm, Dr. Maisch) and eluted via a homemade analytical nano-HPLC column (15 cm × 50 µm; Reprosil-Pur C18-AQ 3 um). The gradient was run from 0% to 50% solvent B (100/0.1 water/formic acid (FA) v/v) in 120 minutes. The nano-HPLC column was drawn to a tip of 5 µm and acted as the electrospray needle of the MS source. The Q-

Exactive mass spectrometer was operated in top10-mode. Parameters were resolution 70,000 at an AGC target value of 3 million maximum fill time of 100 ms (full scan), and resolution 17,500 at an AGC target value of 100,000/maximum fill time of 60 ms for MS/MS at an intensity threshold of 17,000. Apex trigger was set to 1 to 5 seconds, and allowed charges were 2-5. For peptide identification, MS/MS spectra were submitted to the uniprot Homo Sapiens database (UP000005640; Jan 2015; 67911 entries) using Mascot Version 2.2.04 (Matrix Science) with the following settings: 10 ppm and 20 millimass units deviation for precursor and fragment masses, respectively; trypsin was set as enzyme.The fixed modification was carbamidomethyl on Cys. Variable modifications were carbamylation on K and protein N-terminus, oxidation on M and acetylation on the protein N-terminus.

Peptide synthesis and confirmation of identity

Peptides for the confirmation of the sequences identified with mass spectrometry were synthesized according to standard fluorenylmethoxycarbonyl (Fmoc) chemistry using a SyroII peptide synthesizer (MultiSynTech). The integrity of the peptides was confirmed using reverse-phase HPLC and MS. Synthetic peptides were submitted to MS2 on the same instrument and compared with MS2 spectrum from biological samples to confirm the initial identification.

Mice and immunizations

Animal experiments were performed conform national guidelines following approval by the local Ethical Committee for Animal Experimentation. DBA/1 mice were obtained from our breeding colony (originally Harlan) and C57BL/6 mice were purchased from Charles River (8-10 weeks old). For alum immunizations mice received two i.p. injections containing a 1:1 mixture of antigen (100µg) and alhydrogel (Invivogen). Complete Freund's adjuvant (CFA) immunizations were given at the tail base, 100µg antigen in CFA (Difco). Three weeks later a subcutaneous boost was given, 100µg antigen in incomplete Freund's adjuvant (IFA; Sigma-Aldrich). For immunizations in the absence of adjuvant, mice received two injections with 100µg antigen diluted in PBS.

Generation of the anti-CarP monoclonal antibody

Spleen cells of Ca-OVA immunized mice were fused with SP2/0 myeloma cells [1] using PEG1500 (Roche). Hybridoma cells were seeded in ten 96-wells plates and supernatant was tested for anti-CarP positivity by ELISA. SP2/0 cell line was tested for mycoplasma contamination.

Antibody variable region cloning

Antibody variable region genes were amplified from hybridoma cells by RT-PCR, using heavy and light chain variable region specific primers. A pool of degenerate 'forward' primers that

anneal to sequences encoding mouse immunoglobulin leader peptides were used with a pool of 'reverse' primers that anneal to sequences spanning the framework 4–constant region junctions of the heavy and light chains. Alternatively, a pool of degenerate 'forward' primers that anneal to sequences encoding the start of mature mouse heavy and light chain variable regions were used. Restriction sites incorporated in the PCR primers allowed cloning of the amplified variable region genes into mouse IgG2a or mouse kappa mammalian expression vectors.

Cultivating CHOSXE cells

Large scale transient transfections were carried out using UCB's proprietary CHOSXE cell line and electroporation expression platform**.** Cells were maintained in logarithmic growth phase in CDCHO media (LifeTech) supplemented with 2mM Glutamax and agitated at 140rpm in a shaker incubator (Kuhner AG) supplemented with 8% CO_2 at 37°C

Electroporation Transfection

Prior to transfection, the CHOSXE cell numbers and viability were determined using CEDEX cell counter (Innovatis AG) and the required amount of cells ($2x10^8$ cells/ml) were centrifuged at 1400 rpm for 10 minutes. The pelleted cells were washed in Hyclone ^{R} MaxCyte ^{R} buffer (Thermo Scientific) and re-suspended for a further 10 minutes and the pellets were re-suspended at $2x10^8$ cells/ml in fresh buffer. Plasmid DNA, purified using QIAGEN Plasmid *Plus* Giga Kit® was then added at 400ug/ml. Following electroporation using a MaxCyte STX® flow electroporation instrument, the cells were transferred into ProCHO medium (Lonza) containing 2mM Glutamax and antibiotic antimitotic solution and cultured in a wave bag (Cell Bag ™ GE Healthcare) placed on Bioreactor platform set at 37^0C and 5% CO_2 with wave motion induced by 25rpm rocking. 24hr post transfection, a bolus feed was added and the temperature was reduced to 32^0C and maintain for the duration of the culture period (12-14days). At day four, 3mM Sodium butyrate was added to the culture. At day14, the cultures were centrifugation for 30 minutes at 4000rpm and the retained supernatants were filtered through 0.22um SARTO BRAN- P (Millipore) followed by 0.22um Gamma gold filters. Final expression levels were determined by Protein G-HPLC.

Antibody purification

The murine IgG_{2A} antibodies were purified as follows. Following expression a Protein A affinity capture step was performed followed by a preparative size exclusion 'polishing' step. Clarified cell culture supernatants were first 0.22µm sterile filtered and loaded at 4ml/min onto 2x 5ml stacked MabSelect SuRe HiTrap columns (GE Healthcare) equilibrated in PBS pH7.4 (Sigma Aldrich Chemicals).

After loading the columns were washed with PBS pH7.4 and then eluted with 0.1M Sodium Citrate pH3.4. The elution was followed by absorbance at 280nm, the elution peak collected, then neutralised with 1/5th volume of 2M Tris/HCl pH8.5. The neutralized samples were concentrated using Amicon Ultra-15 concentrators with a 30kDa molecular weight cut off membrane and centrifugation at 4000xg in a swing out rotor. Concentrated samples were applied to an XK26/60 Superdex200 column (GE Healthcare) equilibrated in PBS, pH7.4. The column was developed with an isocratic gradient of PBS, pH7.4 at 2.6ml/min respectively. Fractions were collected and analyzed by size exclusion chromatography on a TSK gel G3000SWXL; 5µm, 7.8 X 300mm column developed with an isocratic gradient of 0.2M phosphate, pH7.0 at 1ml/min, with detection by absorbance at 280nm. Selected monomer fractions were pooled. Final samples were assayed; for concentration by A280 Scanning UV-visible spectrophotometer (Cary 50Bio); for % monomer by size exclusion chromatography on a TSK gel G3000SWXL; 5µm, 7.8 X 300mm column developed with an isocratic gradient of 0.2M phosphate, pH7.0 at 1ml/min, with detection by absorbance at 280nm; by reducing and non-reducing SDS-PAGE run on 4-20% Tris-Glycine 1.5mm gels (Novex) at 50mA (per gel) for 53minutes; and for endotoxin by Charles River's EndoSafe® Portable Test System with Limulus Amebocyte Lysate (LAL) test cartridges.

Statistics

Prism7 (GraphPad) or IBM SPSS Statistics23 was used for statistical testing. Statistical differences in inhibition experiments were determined by the Wilcoxon signed-rank test. Differences in antibody levels between subjects and controls were determined by the Mann-Whitney U test. Differences in antibody positivity were determined by Pearson's chi-squared test. Spearman's rank test was performed to evaluate correlations.

Results

Anti-CarP antibodies of RA patients recognize multiple carbamylated antigens.

To characterize the antigen recognition profile of human anti-CarP antibodies, we studied antibody reactivity against a set of five different carbamylated proteins; fetal calf serum (FCS), human serum albumin (HSA), human prothrombin (ProT), bovine histone H1 (H1) and bovine myelin basic protein (MBP). As depicted in Figure 1B and Figure 2A, serum samples from 160 RA patients showed increased recognition of multiple carbamylated antigens as compared to serum from healthy individuals (n=40) (Mann-Whitney U test, $p<0.001$ for each test). Although the overall number of RA patients displaying antibody reactivity towards these five different antigens is similar (ranging from 39% till 58%) (Figure 1C), the antigen recognition profile among individuals differs. In sera of healthy individuals, anti-CarP antibody reactivity, when present, is limited to only one or two carbamylated antigens, while up to 5 out of 5 carbamylated protein antigens are recognized by 24% of RA patients (Figure 1D). Furthermore, antibody levels correlate with the total number of proteins recognized (Figure 1E and Figure 2B). A strong correlation is observed between the mutual recognition of several carbamylated antigens (Figure 1F). Together, these data show that anti-CarP antibodies have a broad antigen recognition profile that correlates with antibody levels. Within this broad antibody response, both self- and foreign carbamylated antigens can be recognized.

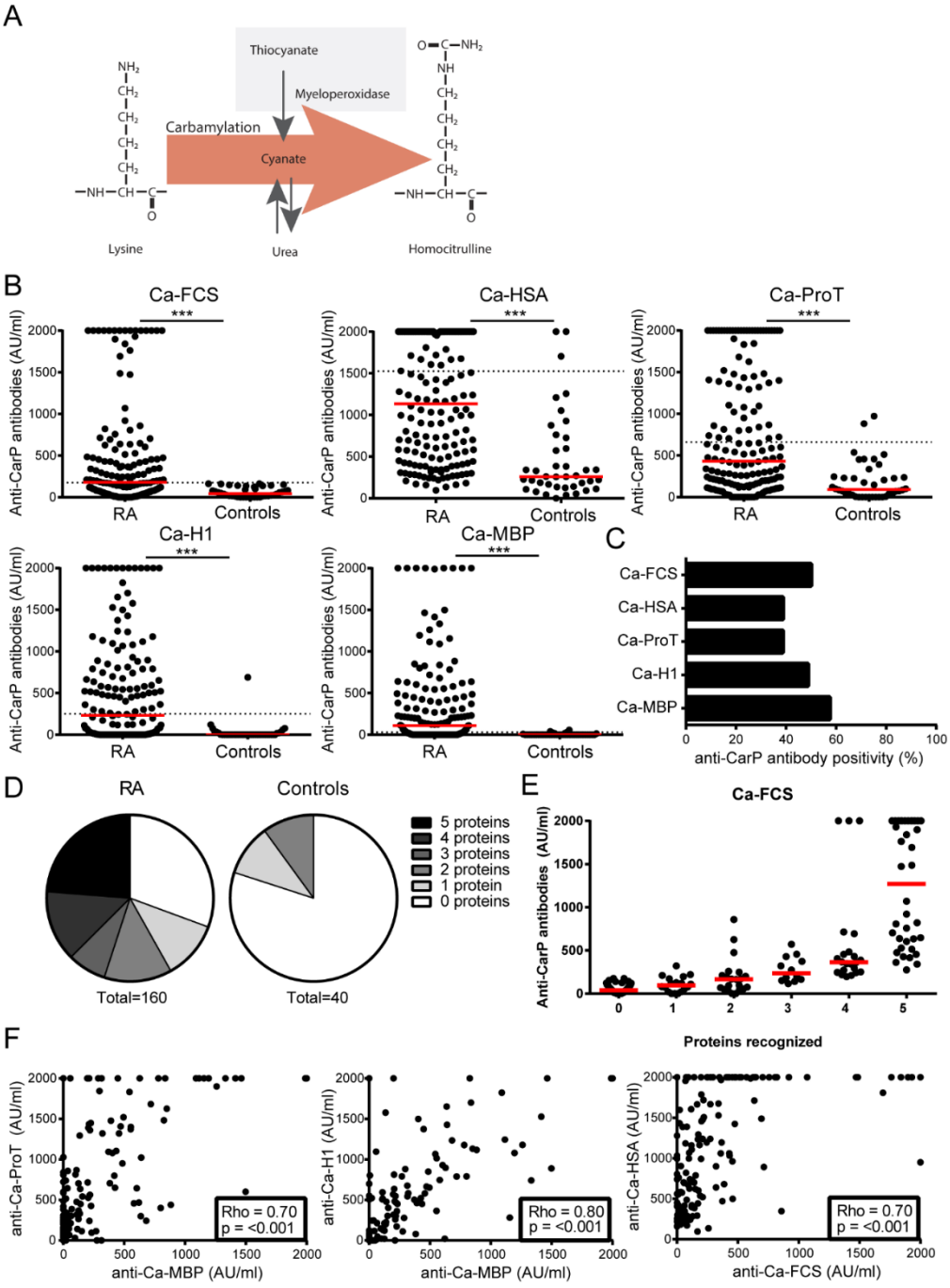

A
Thiocyanate
Myeloperoxidase
Carbamylation
Cyanate
Urea
Lysine
Homocitrulline
B
Ca-FCS
Ca-HSA
Ca-ProT
Ca-H1
Ca-MBP
Anti-CarP antibodies (AU/ml)
***
RA
Controls
C
Ca-FCS
Ca-HSA
Ca-ProT
Ca-H1
Ca-MBP
anti-CarP antibody positivity (%)
D
RA
Controls
5 proteins
4 proteins
3 proteins
2 proteins
1 protein
0 proteins
Total=160
Total=40
E
Ca-FCS
Anti-CarP antibodies (AU/ml)
Proteins recognized
F
anti-Ca-ProT (AU/ml)
anti-Ca-MBP (AU/ml)
Rho = 0.70
p = <0.001
anti-Ca-H1 (AU/ml)
Rho = 0.80
p = <0.001
anti-Ca-HSA (AU/ml)
anti-Ca-FCS (AU/ml)
Rho = 0.70
p = <0.001

Figure 1. Characterization of anti-CarP antibody reactivities in RA patients. (A) During carbamylation a lysine residue is converted into a homocitrulline residue trough a chemical reaction with cyanate. Levels of cyanate are in equilibrium with urea and can be increased, for example, during kidney disease. Cyanate levels can also be elevated during inflammation by the action of myeloperoxidase. (B) Anti-CarP antibody reactivities against five carbamylated and non-modified counterparts were measured by ELISA in 160 RA patients and 40 healthy controls. A standard serum pool was used to calculate the arbitrary units. The represented value was calculated by subtracting the non-modified antigen reactivity from the carbamylated antigen reactivity. The dotted line represents the cut-off while the continuous line represents the median. Statistical differences were determined by the Mann-Whitney U test (***: $p < 0.001$). (C) Percentages of anti-CarP antibody positivity for all five antigens in RA patients. (D) Fractions of patients that display antibody reactivity towards multiple carbamylated antigens are shown for RA patients and controls. (E) Correlation between the amount of antigens recognized by individual serum samples and the anti-Ca-FCS antibody levels. The small continuous line represents the median. Reactivity towards Ca-FCS is shown as an example, Spearman rank test, $P<0.001$ for all carbamylated antigens. (F) The correlation between two anti-CarP antibody reactivities is shown for different carbamylated antigens. The spearman rank test was carried out to determine the degree of statistical correlation. Anti-CarP; anti-carbamylated protein, AU/ml; arbitrary units per milliliter, RA; rheumatoid arthritis, Ca-; carbamylated, FCS; Fetal Calf Serum, HSA; human serum albumin, ProT; prothrombin, H1; H1 Histones, MBP; myelin basic protein.

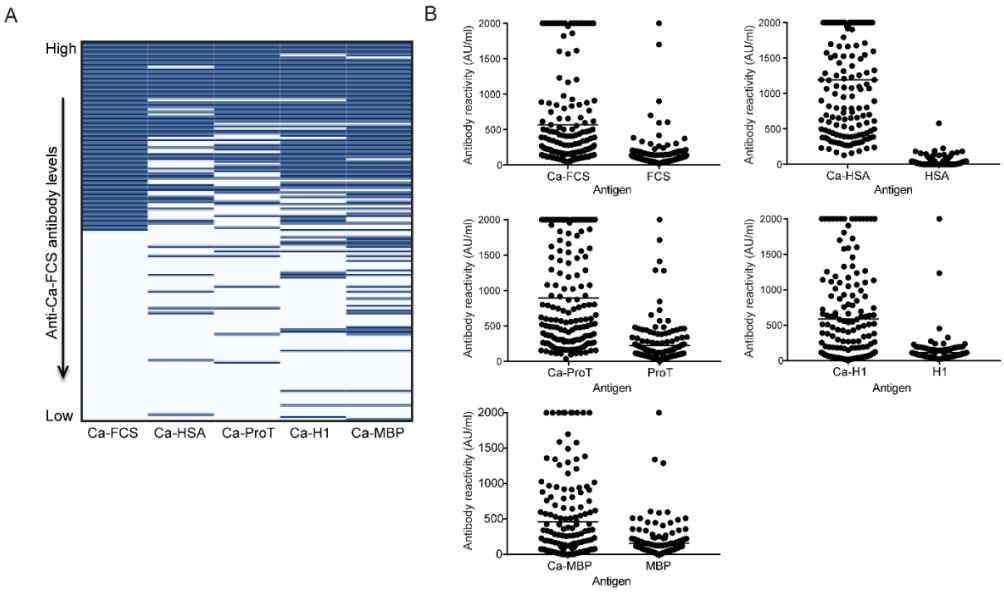


Figure 2. Anti-CarP antibodies of RA patients recognize multiple carbamylated antigens. (A) Anti-CarP antibody reactivities against five carbamylated and non modified counterparts were measured by ELISA. Differential binding of RA sera to the carbamylated proteins compared to the unmodified proteins is shown for 160 RA patients. AU/ml; arbitrary units per milliliter, Ca-; carbamylated, FCS; Fetel Calf Serum, Fib; fibrinogen, HSA; human serum albumin, ProT; prothrombin, H1; H1 Histones, MBP; myelin basic protein. (B) Anti-CarP antibody positivity sorted by anti-Ca-FCS antibody levels. An overview of anti-CarP antibody binding towards five different carbamylated antigens is shown for 160 RA patients. The list was sorted on anti-Ca-FCS antibody levels, showing the highest anti-Ca-FCS antibody levels on top and the lowest levels at the bottom. The dark boxes indicate positivity for that particular antigen, while a white box indicates that a sample was negative. Ca-; carbamylated, FCS; Fetal Calf Serum, HSA; human serum albumin, ProT; prothrombin, H1; H1 Histones, MBP; myelin basic protein.

Human anti-CarP antibodies are highly cross-reactive.

Since anti-CarP antibodies recognize a variety of carbamylated antigens and a high correlation was observed between reactivities, we next investigated whether antibody cross-reactivity could explain these features. Cross-reactivity was determined by inhibition assays using carbamylated proteins or their unmodified counterparts. Titrations and control experiments were performed to ensure non-saturating conditions for sera and inhibitors (Figure 3A-B). Inhibition assays employing the same protein used as antigen and inhibitor acted as positive control. Successful signal inhibition is observed for each of the five antigens (FCS, HSA, ProT, H1 and MBP) using carbamylated inhibitors, while no inhibition is observed for their non-carbamylated counterparts (Figure 3C). Next ten serum samples both reactive to Ca-MBP and Ca-ProT were selected for subsequent inhibition experiments. Binding of anti-Ca-MBP antibodies could be inhibited by incubation with Ca-ProT (Figure 4A and Figure 3D), unlike incubation with unmodified ProT. To characterize the anti-CarP antibody cross-reactivity profile in more detail, two representative serum samples containing antibodies reactive towards all five antigens were studied. In both samples, each carbamylated and non-carbamylated antigen was used to inhibit all five carbamylated antigens (Figure 4B). Most of the carbamylated inhibitors can interfere with antibody binding, although differences between samples and inhibitors exist. Non-carbamylated counterparts do not display an extensive inhibition profile although some inhibition was observed for H1, possibly due to the presence of other PTM on histones (26). Altogether, we observed that carbamylated proteins are able to interfere with antibody binding to other unrelated carbamylated proteins, indicating that anti-CarP antibody cross-reactivity is present in RA patients.

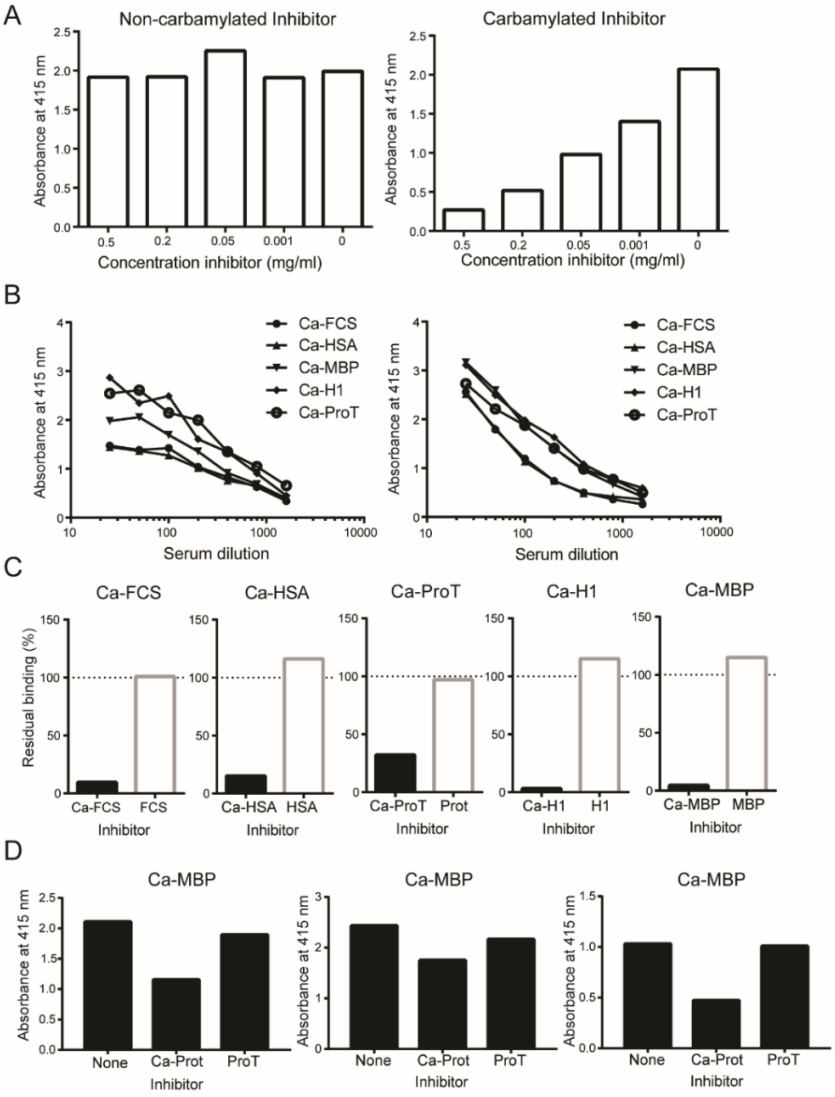


Figure 3. Titration and control experiments for the inhibition studies. (A) Representative figure of an inhibition assay in which the plate was coated with Ca-FCS and Ca-FCS (left) or FCS (right) was used as an inhibitor in different concentrations. (B) Titration assay of two RA serum samples containing antibodies reactive to all five carbamylated antigens. (C) Representative results of inhibition assays employing the same proteins as both antigen and inhibitor, accompanied by the non-modified protein inhibitor as well. The dotted line indicates the anti-CarP antibody reactivity without inhibition. (D) Three examples of anti-Ca-MBP antibody binding when inhibited with no antigen, Ca-ProT or ProT. Anti-CarP; anti-carbamylated protein, AU/ml; arbitrary units per milliliter, RA; rheumatoid arthritis, Ca-; carbamylated, FCS; Fetel Calf Serum, Fib; fibrinogen, HSA; human serum albumin, ProT; prothrombin, H1; H1 Histones, MBP; myelin basic protein.

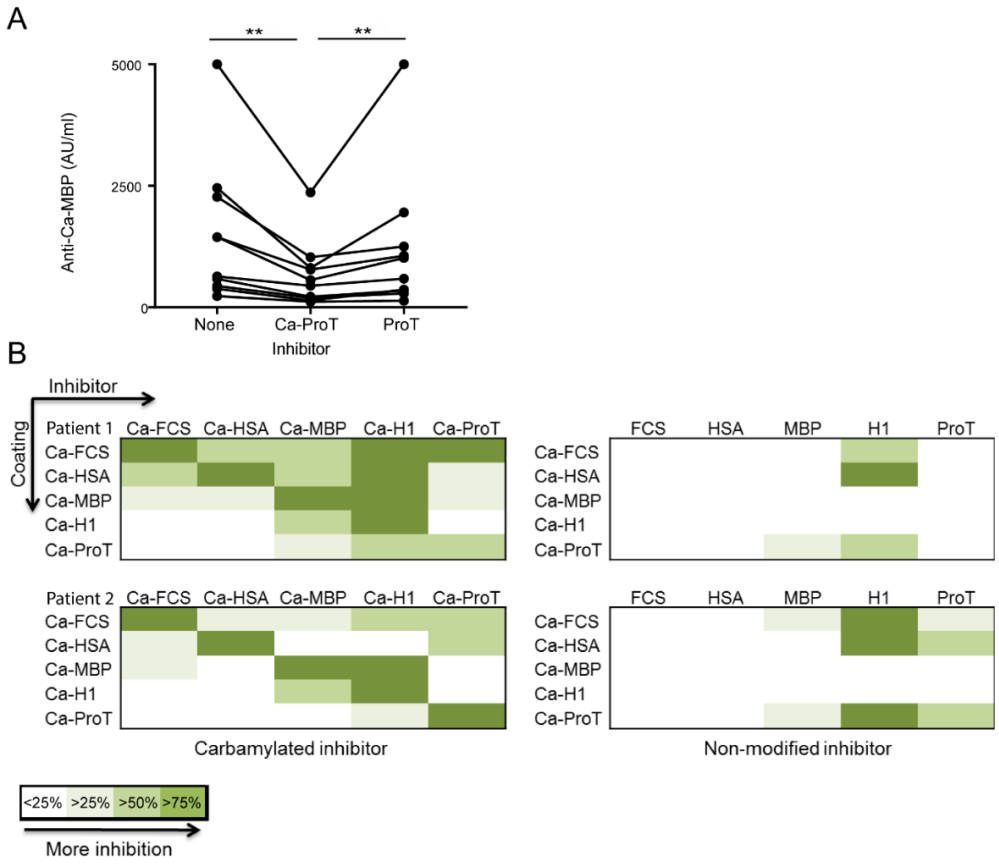


Figure 4. Anti-CarP antibodies are highly cross-reactive. (A) Ten serum samples containing both anti-Ca-MBP and anti-Ca-ProT antibodies were used for inhibition experiments. Anti-Ca-MBP antibody binding was measured with or without the presence of Ca-ProT (0.2mg/ml) or ProT (0.2mg/ml) as inhibitor. Statistical differences were determined by a Wilcoxon signed-rank test (**: P<0.01). (B) An overview of the cross-reactivity of anti-CarP antibodies is depicted. Inhibition assays by ELISA were carried out to determine the degree of cross-reactivity, using two representative serum samples containing antibodies against five carbamylated antigens. Plates were coated with one protein (indicated vertically) and binding of the antibodies towards this coating was inhibited with another, or the same protein (indicated horizontally). Inhibition was calculated as a percentage of the antibody binding without inhibition. The blank was subtracted before calculating. The darkest color indicates an inhibition of more than 75%, followed by an inhibition of more than 50% and the light color indicates an inhibition of more than 25%. The white blocks indicate that the inhibition is less than 25%. Ca-; carbamylated, FCS; Fetal Calf Serum, HSA; human serum albumin, ProT; prothrombin, H1; H1 Histones, MBP; myelin basic protein.

Carbamylated self-proteins are present in RA synovial tissue.

The cross-reactive nature of anti-CarP antibodies suggests that these antibodies might react to a variety of carbamylated proteins present in target tissue. However, little is known about the presence of carbamylated proteins within affected tissue of RA patients. Therefore, we aimed to identify carbamylated self-proteins in synovial tissue of two RA patients by mass spectrometry. From a list of potential hits, four peptides from carbamylated human albumin were selected for further analysis: VFDEF**k**PLVEEPQNLIK**,** **k**LVAASQAALGL, **k**VPQVSTPTLVEVSR, and ADDKETcFAEEG**k**K. The bold, underlined non-capital k, indicates the homocitrulline residue. Three of these four carbamylation sites could be identified in both patients. A representative MS-spectrum of carbamylated albumin-derived peptide VFDEF**k**PLVEEPQNLIK is depicted in Figure 5. Importantly, synthetic peptides with the same sequence displayed highly similar MS-spectra confirming the correct identification of these peptides. MS-spectra of the other albumin-derived peptides are depicted in Figure 6A-C. Other verified proteins in which carbamylation was detected in RA patients include several collagens, fibronectin, fibromodulin, albumin and Sushi-repeat containing protein SRPX2. Similar carbamylated proteins could be detected in the osteoarthritic joint (data not shown). Although, the extent of carbamylation was not quantified, these results indicate that carbamylated self-proteins, are present locally in the synovial compartment of RA patients.

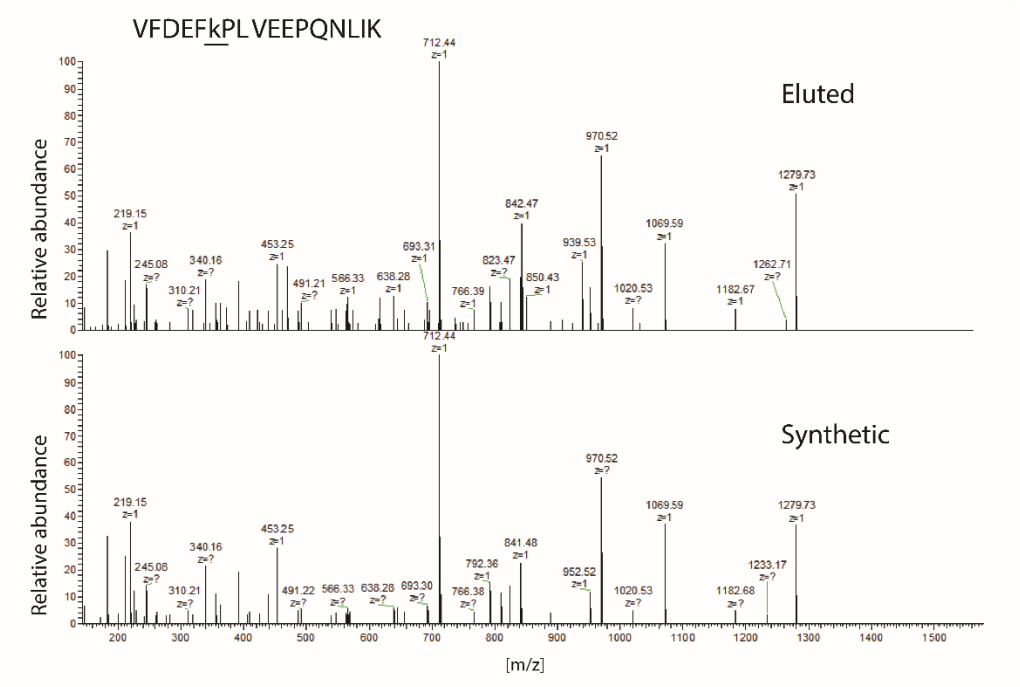
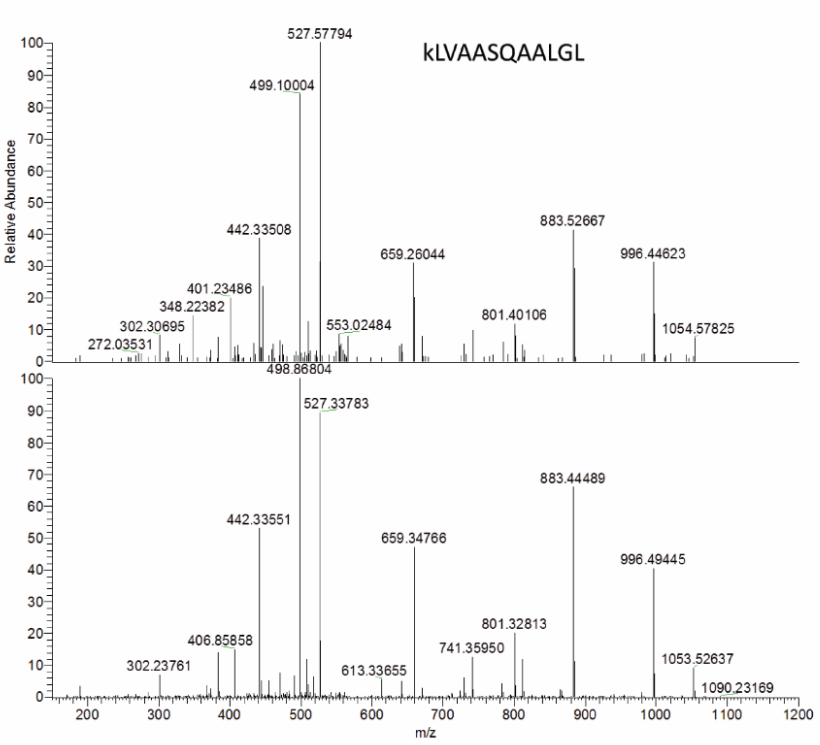
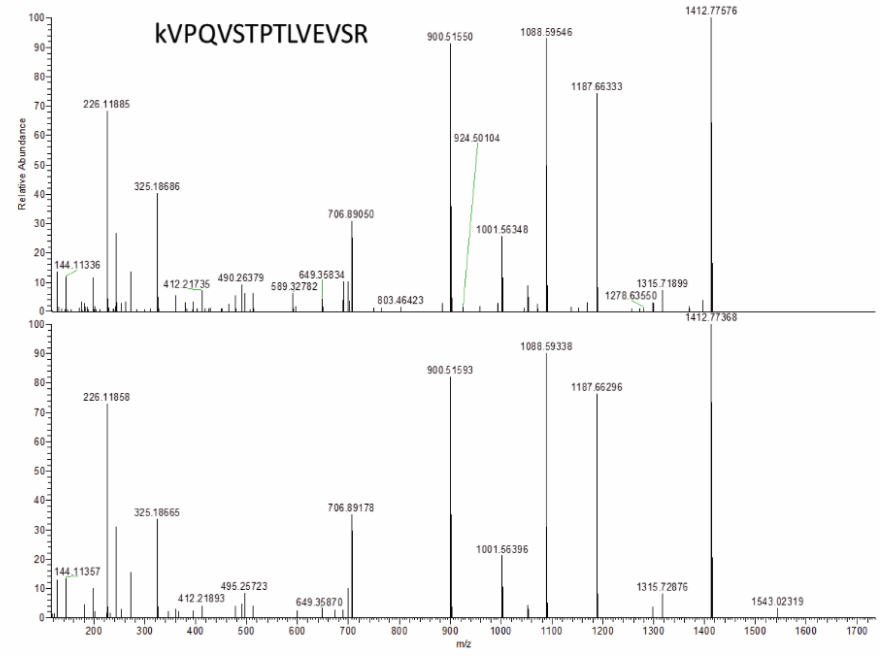


Figure 5. Identification of carbamylated albumin in RA synovial tissue. Tandem mass spectrometry (MS2) spectrum from eluted VFDEF**k**PLVEEPQNLIK peptide (upper panel) derived from carbamylated albumin identified in RA synovial tissue. The synthetic VFDEF**k**PLVEEPQNLIK peptide (lower panel) was submitted to MS2 on the same instrument. The bold, non-captical k, indicates the position of homocitrulline residue.

A


kLVAASQAALGL
Relative Abundance
m/z
527.57794
499.10004
442.33508
883.52667
659.26044
996.44623
401.23486
348.22382
801.40106
302.30695
553.02484
1054.57825
272.03531
498.86804
527.33783
883.44489
442.33551
659.34766
996.49445
801.32813
406.85858
741.35950
302.23761
613.33655
1053.52637
1090.23169

B


kVPQVSTPTLVEVSR
Relative Abundance
m/z
1412.77576
900.51550
1088.59546
1187.66333
226.11885
924.50104
325.18686
706.89050
1001.56348
144.11336
412.21735
490.26379
649.35834
589.32782
803.46423
1315.71899
1278.63550
1412.77368
1088.59338
900.51593
226.11858
1187.66296
325.18665
706.89178
1001.56396
144.11357
495.25723
412.21893
649.35870
1315.72876
1543.02319

C

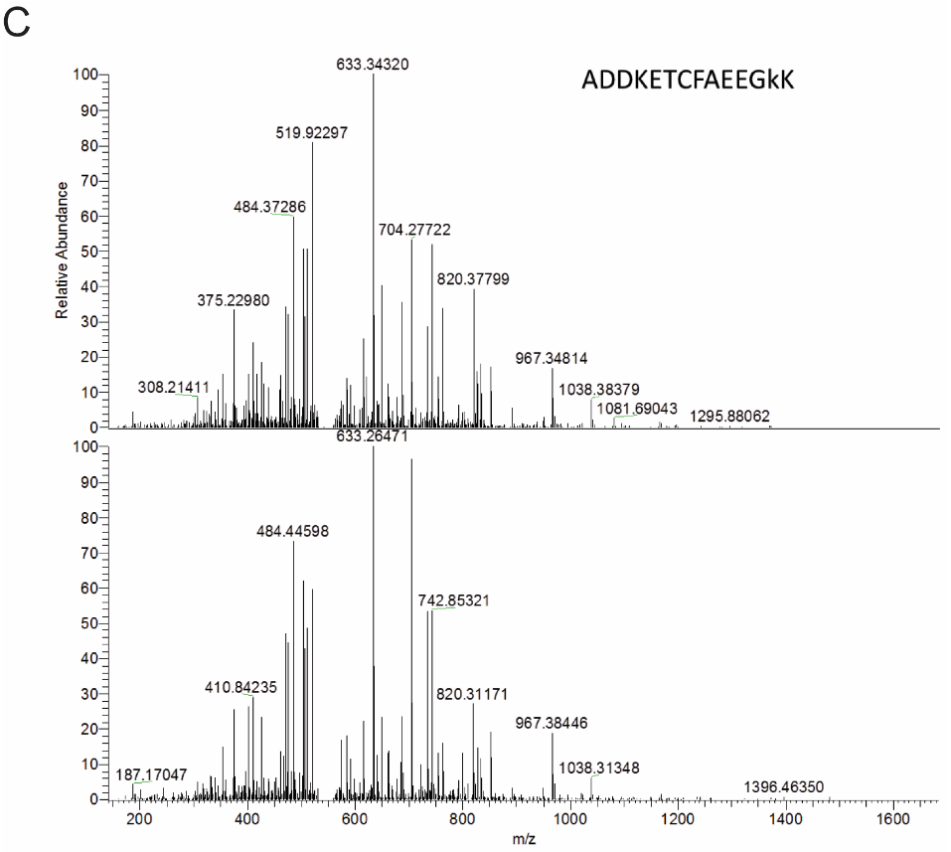


Figure 6. Additional carbamylated albumin peptides identified in the synovial compartment of RA patients. Additional peptides identified in the synovial compartment in RA. MS/MS spectra of peptides derived from carbamylated albumin identified in the RA synovial compartment (upper panel). The synthetic (lower panel) peptide was submitted to MS/MS on the same instrument. The non-captical k, indicates the position of the identified homocitrulline residue. (A) Peptide kLVAASQAALGL, (B) Peptide kVPQVSTPTLVEVSR and (C) Peptide ADDKETcFAEEGkK.

Immunization with carbamylated foreign proteins induces anti-CarP antibodies recognizing carbamylated foreign and self-antigens.

As anti-CarP antibodies from RA patients can recognize both carbamylated self- and non-self proteins, we next investigated whether a carbamylated foreign antigen can facilitate a breach of B cell tolerance towards carbamylated self. Therefore, we immunized mice with Ca-OVA or native OVA in aluminum hydroxide (alum) as a protein free adjuvant. As depicted in Figure 7A and Figure 8A, immunization with both OVA and Ca-OVA results in the induction of a strong antibody response recognizing both modified and non-modified OVA. We subsequently analyzed whether murine anti-CarP-antibodies were cross-reactive by determining antibody reactivity to another carbamylated foreign protein, Ca-FCS (Figure 7B). In this setting, antibody reactivity to the OVA backbone will not be detected. Sera from OVA-immunized mice do not react to Ca-FCS, whereas sera from Ca-OVA immunized mice do contain antibodies reactive to Ca-FCS.

To determine whether AMPAs induced by PTM foreign proteins can cross-react with self-proteins, we examined whether sera from Ca-OVA immunized mice contained antibodies recognizing carbamylated mouse Albumin (mAlb) and fibrinogen (mFib). As depicted in Figure 4C, both Ca-mAlb and Ca-mFib are recognized by sera from Ca-OVA-immunized mice but not by sera from OVA-immunized control animals. Importantly, unmodified mAlb or mFib are not recognized by sera from Ca-OVA-immunized mice. These data show that auto-reactive AMPA-responses can be induced by exposure to carbamylated foreign proteins. These findings were not confined to foreign antigens, as also immunization with carbamylated self–proteins (mAlb and mFib) induced, a cross-reactive anti-CarP antibody response (Figure 7D-E and supplementary Figure 8B-D). Nonetheless, these data are important as they show that even in the context of a highly immunogenic 'foreign' antigen the immune response also specifically recognizes small PTMs as evidenced by the presence of anti-CarP antibody responses.

Because of the high structural homology between citrulline and homocitrulline we next determined whether murine anti-CarP antibodies could recognize citrullinated antigens as well. However, despite minor difference in chemical structure, no binding to Cit-Fib or Cit-FCS was detectable using anti-CarP antibody containing sera from Ca-OVA immunized mice (Figure 7F). In contrast, ACPA-containing sera from RA patients do recognize these citrullinated antigens. To examine the potential immunogenicity of carbamylated foreign proteins in absence of adjuvants, we next immunized mice with Ca-OVA in PBS. Interestingly, significant antibody responses against carbamylated self-proteins were induced (Figure 7G), showing that immunization with carbamylated foreign proteins in absence of adjuvant also results in a cross-reactive B cell response against modified self-proteins. These findings further support the notion that AMPA responses can be generated by exposure to carbamylated foreign antigens.

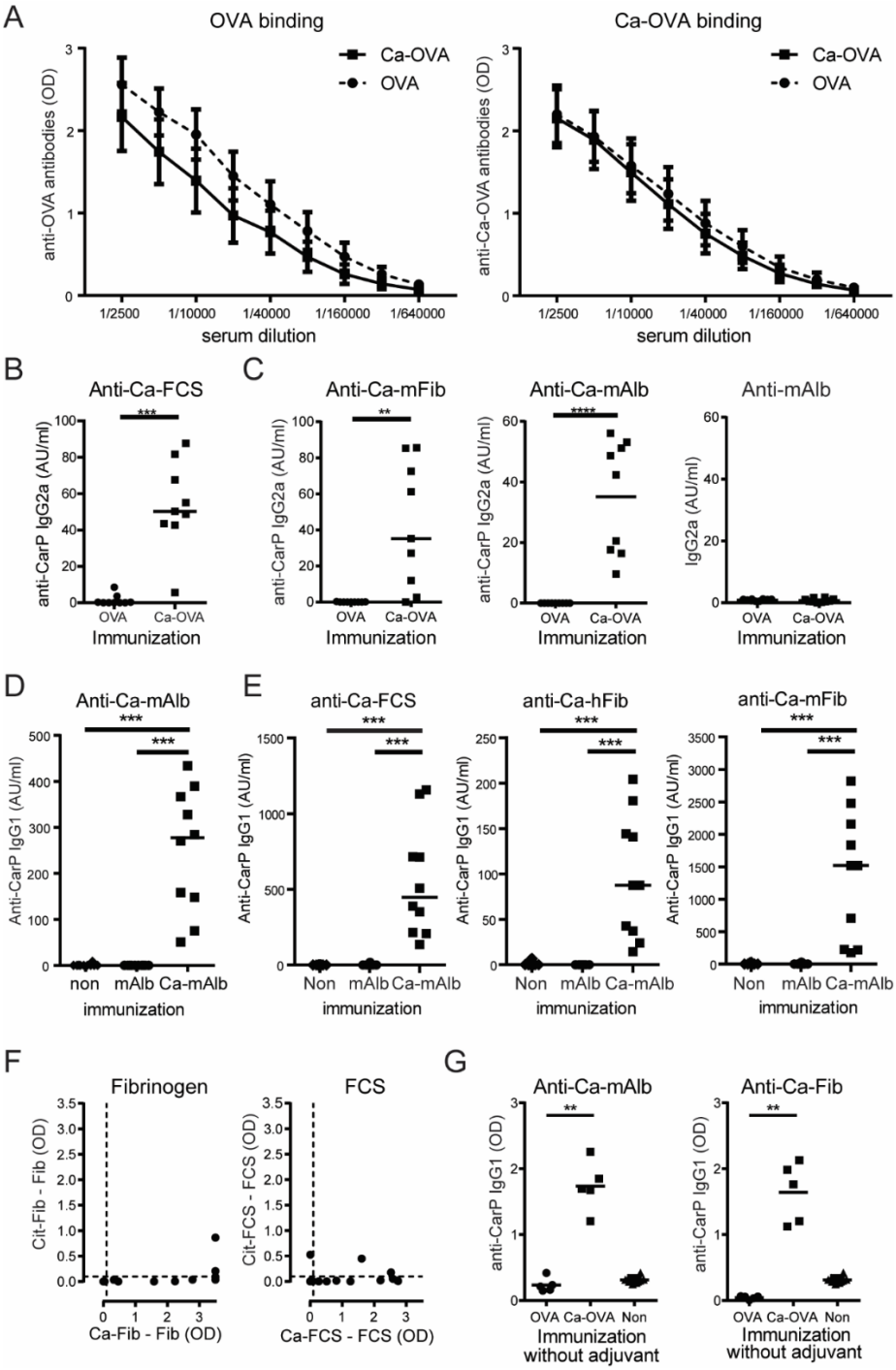

A
OVA binding
Ca-OVA binding
Ca-OVA
OVA
anti-OVA antibodies (OD)
anti-Ca-OVA antibodies (OD)
1/2500
1/10000
1/40000
1/160000
1/640000
serum dilution
B
Anti-Ca-FCS
anti-CarP IgG2a (AU/ml)
OVA
Ca-OVA
Immunization
C
Anti-Ca-mFib
Anti-Ca-mAlb
Anti-mAlb
IgG2a (AU/ml)
D
Anti-Ca-mAlb
Anti-CarP IgG1 (AU/ml)
non
mAlb
Ca-mAlb
immunization
E
anti-Ca-FCS
anti-Ca-hFib
anti-Ca-mFib
Non
F
Fibrinogen
FCS
Cit-Fib - Fib (OD)
Ca-Fib - Fib (OD)
Cit-FCS - FCS (OD)
Ca-FCS - FCS (OD)
G
Anti-Ca-mAlb
Anti-Ca-Fib
anti-CarP IgG1 (OD)
Immunization
without adjuvant

Figure 7. Carbamylated foreign proteins can induce cross-reactive anti-CarP antibodies. (A) Mice were immunized with foreign antigens ovalbumin (OVA) or Ca-OVA. Sera from immunized mice, OVA (circles) and Ca-OVA (squares), was analyzed for binding towards OVA (left panel) and anti-Ca-OVA (right panel) by ELISA (n=10). (B) Antibody reactivity towards Ca-FCS from mice immunized with OVA (circles) and Ca-OVA (squares) was determined by ELISA. Representative data from 3 experiments are shown. Each dot represents data from one mouse (n= 10, *** $p<0.001$ and ** $p<0.01$, Mann-Whitney U test). (C) Antibody reactivity towards Ca-mFib (left panel), Ca-mAlb (middle panel) and native mAlb (right panel) from immunized mice, OVA (circles) and Ca-OVA (squares). Representative data from 3 experiments are shown (n= 10, Mann-Whitney U test, *** $p<0.001$). (D) Mice were immunized with a carbamylated self-antigen, mouse albumin (Ca-mAlb), or native albumin (mAlb) in aluminum hydroxide. Sera from immunized mice (Ca-mAlb (depicted as squares), mAlb (depicted as circles) and non-immunized mice (depicted as triangles) was analyzed for reactivity towards Ca-mAlb. Representative data from 3 experiments are shown. Each dot represents data from one mouse. Statistical difference was determined by the Mann-Whitney U test (n= 10, *** $p<0.001$). (E) Sera of (Ca-)mAlb immunized mice was analyzed for reactivity towards Ca-FCS (left panel), Ca-human fibrinogen (Ca-hFib) (middle panel) and Ca-mouse fibrinogen (Ca-mFib) (right panel). Sera from Ca-mAlb (depicted as squares), mAlb (depicted as circles) and non- immunized mice (depicted as triangles) were analyzed by ELISA. Representative data from 3 experiments are shown. Each dot represents data from one mouse. (n= 10, *** $p<0.001$, Mann-Whitney U test). (F) Correlation between antibody reactivity of Ca-OVA immunized mice towards carbamylated human fibrinogen (Ca-Fib) and citrullinated human fibrinogen (Cit-Fib) (left panel) and correlation between antibody reactivity towards Ca-FCS versus citrullinated FCS (Cit-FCS) (right panel) (n=10, Spearman rank test). (G) Mice were immunized with foreign antigens ovalbumin (OVA) or Ca-OVA in the absence of adjuvant. Sera from immunized mice OVA (circles), Ca-OVA (squares) and non-immunized mice (triangles) was analyzed for binding towards carbamylated self-proteins, Ca-mAlb (left panel) and Ca-mFib (right panel) (n= 5, Mann-Whitney U test, ** $p<0.01$).

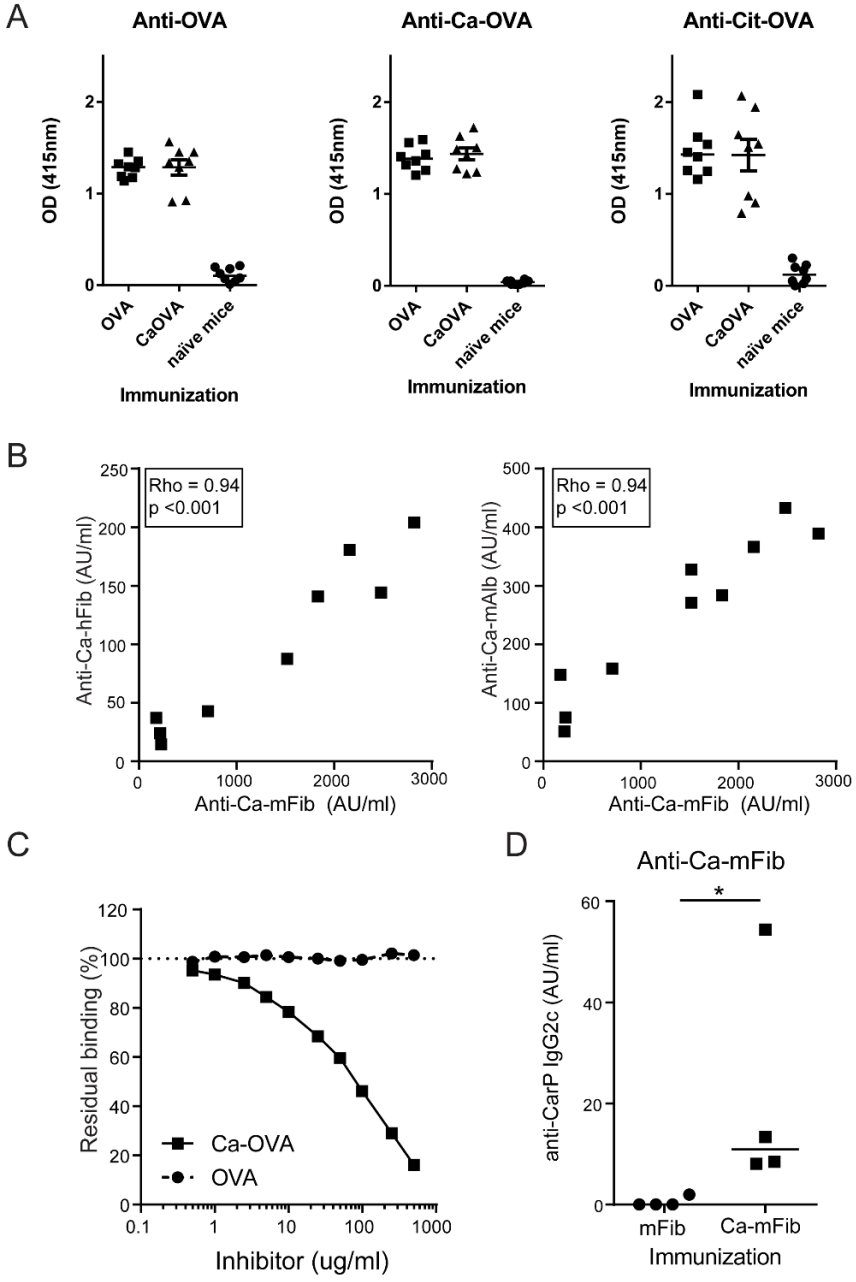


Figure 8. Immunization with carbamylated proteins induces anti-CarP antibodies. (A) Sera from mice immunized with carbamylated ovalbumin (Ca-OVA) (squares, n=8) or non-modified OVA (triangles, n=8) and sera from non-immunized mice were tested for antibody reactivity towards OVA (left panel), Ca-OVA (middle panel) and citrullinated ovalbumin (Cit-OVA) (right panel). (B) Mice were immunized

with a carbamylated self-antigen, mouse albumin (Ca-mAlb) in alum. Correlations between antibody reactivity towards Ca-mFib (mouse fibrinogen) versus Ca-hFib (human fibrogen) (left panel) and Ca-mAlb versus Ca-mFib (right panel) of Ca-mAlb immunized mice are shown. Statistical correlation was determined by the Spearman's rank test (n=10, $p<0.001$). (C) Sera from Ca-mAlb immunized mice were pre-incubated with either Ca-OVA (squares) or OVA (circles) and subsequently analysed for residual binding using a Ca-hFib ELISA (n=10). (D) Mice were immunized with carbamylated self-antigen Ca-mFib or non-modified mFib emulsified in Freund's adjuvant. Sera from mice immunized with mFib (circles) and Ca-mFib (squares) were harvested and analysed for binding towards Ca-mFib by ELISA (n= 4, Mann-Whitney U test, * $p<0.05$)

Monoclonal anti-CarP antibodies show a similar pattern of cross-reactivity towards carbamylated foreign and self-proteins.

To confirm the cross-reactive nature of anti-CarP antibody responses, we generated a murine anti-CarP monoclonal from a mouse immunized with Ca-OVA. As depicted in Figure 9A, this monoclonal antibody binds both carbamylated foreign- and self-proteins. We observe a significant correlation between the monoclonal antibody binding to Ca-FCS, Ca-OVA and Ca-Fib (Figure 9B) confirming its cross-reactive nature. Thus, as observed for polyclonal anti-CarP antibodies from mice immunized with a foreign antigen, strong cross-reactivity is observed towards different carbamylated foreign and self-proteins at the monoclonal antibody level, confirming that self-reactive AMPAs can be induced by exposure to foreign PTM proteins.

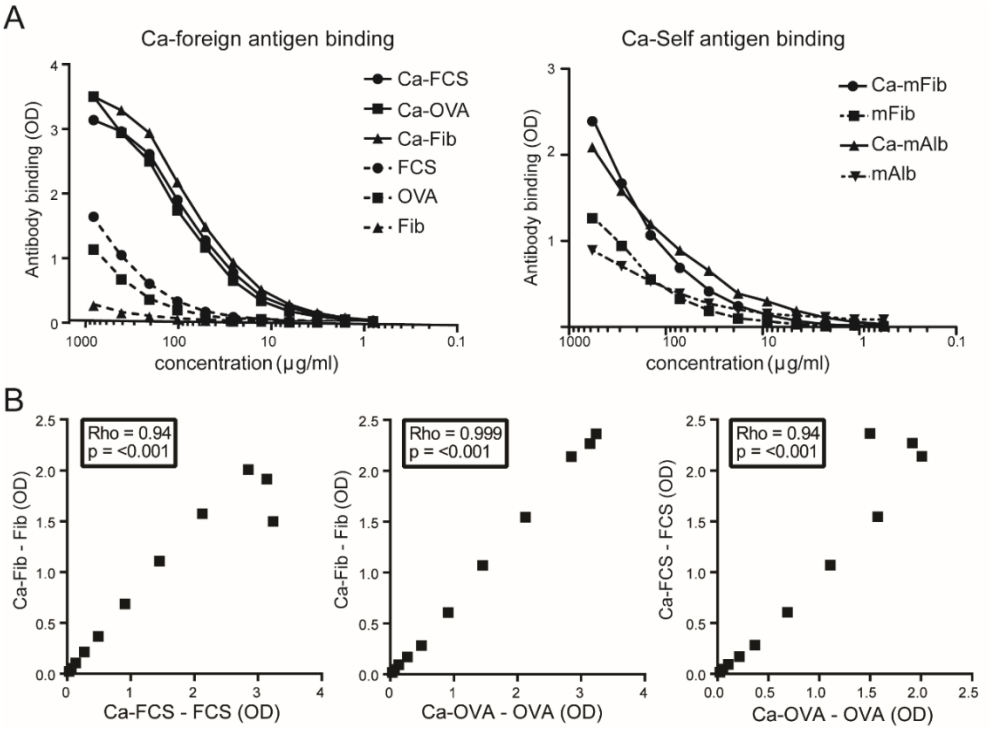


Figure 9. Monoclonal antibodies are highly specific and cross-reactive towards carbamylated foreign and self-antigens. (A) Binding of a murine anti-CarP monoclonal antibody towards carbamylated foreign proteins; Ca-FCS, Ca-Ovalbumin (Ca-OVA), Ca-human Fibrinogen (Ca-Fib) and non-modified counterparts was tested using ELISA (left panel). Reactivity of the anti-CarP monoclonal antibody towards carbamylated self-proteins; Ca-mouse fibrinogen (mFib) and Ca-mouse Albumin (mAlb) and non-modified counterparts was measured by ELISA (right panel). **(B)** The correlation between anti-CarP antibody reactivity towards Ca-Fib and Ca-FCS is shown in the left panel (rho=0.999) and for Ca-Fib compared to Ca-OVA is depicted in the middle panel (rho=0.936). Correlation between anti-CarP antibody reactivity towards Ca-OVA and Ca-FCS is depicted in the right panel (rho=0.936). The spearman rank test was carried out to determine the degree of statistical correlation (n=11).

Discussion

A key characteristic of RA is the occurrence of autoantibodies against PTM proteins (2, 8-10). Here, we report that post-translational modification of foreign proteins, in particular carbamylation, represents one way in which immune tolerance at the B cell level towards self can be broken. In RA patients we found that anti-CarP antibodies present within one serum sample are cross-reactive towards different carbamylated proteins, including foreign and self-proteins. To study how autoreactive B cell responses against PTM self-proteins can be induced, we used carbamylated model antigens (OVA and mAlb) in mice. Our observations reveal that not only carbamylation of self- but also of foreign proteins is sufficient for a breach of immunological tolerance and the formation of autoreactive anti-CarP antibodies. Previous animal studies showed that immunogenicity of proteins is enhanced upon citrullination (27-29). Likewise, although cross-reactive AMPA responses have been described (9, 10, 30), it has not been demonstrated that AMPA-producing B cells recognizing a particular modified self-protein can be induced by other -unrelated- modified proteins or that immunization with a modified (structurally unrelated) foreign protein leads to the induction of a cross-reactive AMPA-response against self. Clearly, human studies demonstrating this principle are challenging as the autoantibody-inciting events are unknown and difficult to control (31).

Recently, we showed that mice are able to mount an antibody response against carbamylated proteins (23). Therefore, we could now address the question whether exposure of a host to a carbamylated foreign protein can lead to the formation of an autoreactive B cells response. Our data show that anti-CarP autoantibodies can, indeed, be induced by carbamylated foreign antigens. These autoantibodies react, both at the polyclonal- as well as the monoclonal level, to different carbamylated proteins, confirming that anti-CarP antibodies are cross-reactive. This high-level cross-reactivity is likely explaining why carbamylated foreign proteins can induce an autoreactive B cell response, and indicate that the epitope recognized by responding B cells can be present on a variety of proteins, either of self- or non-self origin. In RA, we have shown that anti-CarP antibodies are able to recognize different carbamylated (auto)antigens. Similar findings have been reported for other AMPA responses(9, 10, 30). For example, previous human studies have shown that also ACPA exhibit cross-reactive properties towards different citrullinated self- and foreign antigens (30, 32-36). Interestingly, although citrulline greatly resembles homocitrulline in structure, we were unable to detect an antibody response against citrullinated proteins in mice. Also vaccination with citrullinated proteins did not induce an ACPA response (data not shown). Therefore, we were not able to analyze whether autoreactive ACPA could also be induced by (citrullinated) foreign proteins. Nonetheless, given the cross-reactive properties of ACPA (37-40), it is highly conceivable that similar principles as identified for anti-CarP-antibody responses apply to other classes of AMPA as

well. Although it is unknown how autoantibodies against PTM proteins are generated in humans, it is often speculated that an autoreactive T cell response recognizing such self-proteins is crucial for their appearance.

Clearly, our results are not incompatible with this notion and do not indicate that such T cell help would not contribute the induction of B cell mediated autoimmunity against PTM proteins. However, our results provide first evidence that also T cells recognizing "conventional" foreign antigens could be involved in the induction of AMPA-producing B cell responses that recognize modified self-proteins. So far it is unclear to what extent T cell tolerance is lost in RA as identification of PTM epitopes recognized by autoreactive T cells has been proven difficult. Although T cell responses against PTM self-proteins have been described (41-43), frequencies of citrulline specific T cells are estimated to be 10 times lower as compared to T cell frequencies to recall antigen as approximately 1 in 100,000 CD4 cells have been reported to react with tetramers containing citrullinated peptides compared to 1:10,000 CD4 cells for tetanus toxoid specific T cells (43, 44). Nonetheless, animal studies show that PTM proteins can generate antigen-specific T cell responses (19, 27-29, 45). However, at present it is still unclear to what extent these T cells provide help to autoreactive B cells in human RA. Our data indicate that T cell help required for the generation of isotype-switched AMPA-responses can be provided by T cells directed against foreign antigens (Figure 10).

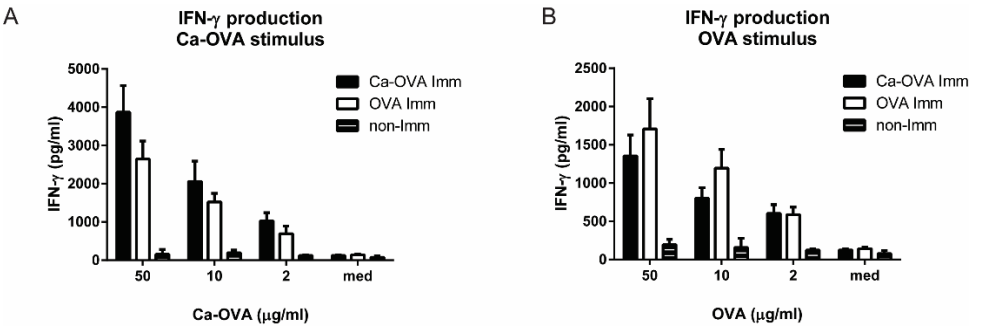


Figure 10. Interferon-γ production by bulk T-cell cultures after stimulation with ovalbumin as foreign antigen. Mice were immunized with carbamylated ovalbumin (Ca-OVA, n=9) (black bars) or non-modified ovalbumin (OVA, n=9) (white bars) in CFA and boosted with the same antigen in IFA. Spleens cells of Ca-OVA, OVA and naïve mice (striped bars, n=2) were in vitro stimulated with D1 cells pulsed with different concentrations of Ca-OVA (A) and OVA (B), or medium. Interferon-γ ELISA was performed as readout for T-cell activation after in vitro stimulation of spleen cells. The samples are pooled data from two independent experiments.

We consider it likely that the initiating event leading to the formation of autoantibodies against carbamylated proteins is not found in the induction of a (T cell) response against carbamylated self-proteins but rather in the induction of immune responses against modified foreign antigens. The only requirement would be that the foreign antigen recognized by the T cells contains PTMs seen by B cells. Such requirement could be met during infection as the conditions to post-translationally modify microbe-derived proteins readily occur during infection. This could, for example, be mediated through release of PAD by neutrophils during netosis (citrullination) (46, 47), the release of myeloid peroxidase leading to enhanced carbamylation or the presence of bacterial-derived acetylated proteins (1, 12, 48). In all these cases, microbe-derived proteins express or can acquire a PTM that can be targeted by responding B cells. These B cells are likely to obtain help from microbe-directed T cells required for further somatic hypermutation. Since self-proteins can also undergo similar PTM, some B cells will conceivably be selected on modified self-proteins leading to the development of a self-reactive B cell response. In this scenario, autoimmunity can emerge without the presence of autoreactive T cells.

These considerations are important for the development of tolerizing protocols aiming to dampen or inactivate putative autoreactive T cells in an antigen-specific fashion. Likewise, they are also of relevance to define the autoimmune inciting antigen as the recognition of a particular antigen by autoreactive B cells or antibodies might not relate to the antigen that was required to induce the B cell response.

In conclusion, our results clearly indicate that carbamylated foreign proteins are able to induce a breach of tolerance at the B cell level leading to the formation of cross-reactive anti-CarP antibodies recognizing modified self-proteins. We consider it likely that anti-CarP B cell responses can result from inflammatory conditions induced for example by infection, as it is conceivable that in such conditions carbamylated foreign proteins are recognized by the responding immune system. The evoking anti-CarP immune response might subsequently cross-react to carbamylated self-proteins that are also expressed in the joints of RA patients thereby possibly contributing to the local inflammatory reaction present in RA.

References

1. Anzilotti C, Pratesi F, Tommasi C, Migliorini P**.** Peptidylarginine deiminase 4 and citrullination in health and disease. Autoimmun Rev. 2010;9(3):158-60.
2. Willemze A, Trouw LA, Toes RE, Huizinga TW**.** The influence of ACPA status and characteristics on the course of RA. Nat Rev Rheumatol. 2012;8(3):144-52.
3. De Rycke L, Peene I, Hoffman IE, Kruithof E, Union A, Meheus L, et al. Rheumatoid factor and anticitrullinated protein antibodies in rheumatoid arthritis: diagnostic value, associations with radiological progression rate, and extra-articular manifestations. Ann Rheum Dis. 2004;63(12):1587-93.
4. Shidara K, Inoue E, Hoshi D, Sato E, Nakajima A, Momohara S, et al. Anti-cyclic citrullinated peptide antibody predicts functional disability in patients with rheumatoid arthritis in a large prospective observational cohort in Japan. Rheumatol Int. 2012;32(2):361-6.
5. Humphreys JH, van Nies JA, Chipping J, Marshall T, van der Helm-van Mil AH, Symmons DP, et al. Rheumatoid factor and anti-citrullinated protein antibody positivity, but not level, are associated with increased mortality in patients with rheumatoid arthritis: results from two large independent cohorts. Arthritis Res Ther. 2014;16(6):483.
6. Huizinga TW, Amos CI, van der Helm-van Mil AH, Chen W, van Gaalen FA, Jawaheer D, et al. Refining the complex rheumatoid arthritis phenotype based on specificity of the HLA-DRB1 shared epitope for antibodies to citrullinated proteins. Arthritis Rheum. 2005;52(11):3433-8.
7. Linn-Rasker SP, van der Helm-van Mil AH, van Gaalen FA, Kloppenburg M, de Vries RR, le Cessie S, et al. Smoking is a risk factor for anti-CCP antibodies only in rheumatoid arthritis patients who carry HLA-DRB1 shared epitope alleles. Ann Rheum Dis. 2006;65(3):366-71.
8. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci U S A. 2011;108(42):17372-7.
9. Thiele GM, Duryee MJ, Anderson DR, Klassen LW, Mohring SM, Young KA, et al. Malondialdehyde-acetaldehyde adducts and anti-malondialdehyde-acetaldehyde antibodies in rheumatoid arthritis. Arthritis Rheumatol. 2015;67(3):645-55.
10. Juarez M, Bang H, Hammar F, Reimer U, Dyke B, Sahbudin I, et al. Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis. Ann Rheum Dis. 2016;75(6):1099-107.
11. Figueiredo CP, Bang H, Cobra JF, Englbrecht M, Hueber AJ, Haschka J, et al. Antimodified protein antibody response pattern influences the risk for disease relapse in patients with rheumatoid arthritis tapering disease modifying antirheumatic drugs. Ann Rheum Dis. 2016.
12. Shi J, van Veelen PA, Mahler M, Janssen GM, Drijfhout JW, Huizinga TW, et al. Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies. Autoimmun Rev. 2014;13(3):225-30.
13. Brink M, Verheul MK, Ronnelid J, Berglin E, Holmdahl R, Toes RE, et al. Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage. Arthritis Res Ther. 2015;17:25.

14. Shi J, van de Stadt LA, Levarht EW, Huizinga TW, Hamann D, van Schaardenburg D, et al. Anti-carbamylated protein (anti-CarP) antibodies precede the onset of rheumatoid arthritis. Ann Rheum Dis. 2014;73(4):780-3.
15. Gan RW, Trouw LA, Shi J, Toes RE, Huizinga TW, Demoruelle MK, et al. Anti-carbamylated protein antibodies are present prior to rheumatoid arthritis and are associated with its future diagnosis. J Rheumatol. 2015;42(4):572-9.
16. Shi J, van de Stadt LA, Levarht EW, Huizinga TW, Toes RE, Trouw LA, et al. Anti-carbamylated protein antibodies are present in arthralgia patients and predict the development of rheumatoid arthritis. Arthritis Rheum. 2013;65(4):911-5.
17. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum. 2003;48(10):2741-9.
18. Nielen MM, van Schaardenburg D, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MH, et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum. 2004;50(2):380-6.
19. Mydel P, Wang Z, Brisslert M, Hellvard A, Dahlberg LE, Hazen SL, et al. Carbamylation-dependent activation of T cells: a novel mechanism in the pathogenesis of autoimmune arthritis. J Immunol. 2010;184(12):6882-90.
20. Burska AN, Hunt L, Boissinot M, Strollo R, Ryan BJ, Vital E, et al. Autoantibodies to posttranslational modifications in rheumatoid arthritis. Mediators Inflamm. 2014;2014:492873.
21. Zavala-Cerna MG, Martinez-Garcia EA, Torres-Bugarin O, Rubio-Jurado B, Riebeling C, Nava A**.** The clinical significance of posttranslational modification of autoantigens. Clin Rev Allergy Immunol. 2014;47(1):73-90.
22. Vossenaar ER, Nijenhuis S, Helsen MM, van der Heijden A, Senshu T, van den Berg WB, et al. Citrullination of synovial proteins in murine models of rheumatoid arthritis. Arthritis Rheum. 2003;48(9):2489-500.
23. Stoop JN, Liu BS, Shi J, Jansen DT, Hegen M, Huizinga TW, et al. Antibodies specific for carbamylated proteins precede the onset of clinical symptoms in mice with collagen induced arthritis. PLoS One. 2014;9(7):e102163.
24. Stoop JN, Fischer A, Hayer S, Hegen M, Huizinga TW, Steiner G, et al. Anticarbamylated protein antibodies can be detected in animal models of arthritis that require active involvement of the adaptive immune system. Ann Rheum Dis. 2015;74(5):949-50.
25. de Rooy DP, van der Linden MP, Knevel R, Huizinga TW, van der Helm-van Mil AH**.** Predicting arthritis outcomes--what can be learned from the Leiden Early Arthritis Clinic? Rheumatology (Oxford). 2011;50(1):93-100.
26. Tessarz P, Kouzarides T**.** Histone core modifications regulating nucleosome structure and dynamics. Nat Rev Mol Cell Biol. 2014;15(11):703-8.
27. Lundberg K, Nijenhuis S, Vossenaar ER, Palmblad K, van Venrooij WJ, Klareskog L, et al. Citrullinated proteins have increased immunogenicity and arthritogenicity and their presence in arthritic joints correlates with disease severity. Arthritis Res Ther. 2005;7(3):R458-67.

28. Hill JA, Bell DA, Brintnell W, Yue D, Wehrli B, Jevnikar AM, et al. Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. J Exp Med. 2008;205(4):967-79.
29. Kinloch AJ, Alzabin S, Brintnell W, Wilson E, Barra L, Wegner N, et al. Immunization with porphyromonas gingivalis enolase induces autoimmunity to mammalian alpha-enolase and arthritis in DR4-IE-transgenic mice. Arthritis and Rheumatism. 2011;63(12):3818-23.
30. Ioan-Facsinay A, el-Bannoudi H, Scherer HU, van der Woude D, Menard HA, Lora M, et al. Anti-cyclic citrullinated peptide antibodies are a collection of anti-citrullinated protein antibodies and contain overlapping and non-overlapping reactivities. Ann Rheum Dis. 2011;70(1):188-93.
31. Lundberg K, Kinloch A, Fisher BA, Wegner N, Wait R, Charles P, et al. Antibodies to Citrullinated alpha-Enolase Peptide 1 Are Specific for Rheumatoid Arthritis and Cross-React With Bacterial Enolase. Arthritis and Rheumatism. 2008;58(10):3009-19.
32. van Beers JJ, Willemze A, Jansen JJ, Engbers GH, Salden M, Raats J, et al. ACPA fine-specificity profiles in early rheumatoid arthritis patients do not correlate with clinical features at baseline or with disease progression. Arthritis Res Ther. 2013;15(5):R140.
33. van der Woude D, Rantapaa-Dahlqvist S, Ioan-Facsinay A, Onnekink C, Schwarte CM, Verpoort KN, et al. Epitope spreading of the anti-citrullinated protein antibody response occurs before disease onset and is associated with the disease course of early arthritis. Ann Rheum Dis. 2010;69(8):1554-61.
34. van Heemst J, Trouw LA, Nogueira L, van Steenbergen HW, van der Helm-van Mil AH, Allaart CF, et al. An investigation of the added value of an ACPA multiplex assay in an early rheumatoid arthritis setting. Arthritis Res Ther. 2015;17:276.
35. Lundberg K, Bengtsson C, Kharlamova N, Reed E, Jiang X, Kallberg H, et al. Genetic and environmental determinants for disease risk in subsets of rheumatoid arthritis defined by the anticitrullinated protein/peptide antibody fine specificity profile. Ann Rheum Dis. 2013;72(5):652-8.
36. Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. PLoS One. 2012;7(5):e35296.
37. Scinocca M, Bell DA, Racape M, Joseph R, Shaw G, et al. Antihomocitrullinated Fibrinogen Antibodies are Specific to Rheumatoid Arthritis and Frequently Bind Citrullinated Proteins/peptides. Journal of Rheumatology. 2014;41(2):270-9.
38. Shi J, Willemze A, Janssen GM, van Veelen PA, Drijfhout JW, Cerami A, et al. Recognition of citrullinated and carbamylated proteins by human antibodies: specificity, cross-reactivity and the 'AMC-Senshu' method. Ann Rheum Dis. 2013;72(1):148-50.
39. Juarez M, Bang H, Hammar F, Reimer U, Dyke B, Sahbudin I, et al. Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis. Annals of the Rheumatic Diseases. 2016;75(6):1099-107.
40. Reed E, Jiang X, Kharlamova N, Ytterberg AJ, Catrina AI, Israelsson L, et al. Antibodies to carbamylated alpha-enolase epitopes in rheumatoid arthritis also bind citrullinated epitopes and are largely indistinct from anti-citrullinated protein antibodies. Arthritis Research & Therapy. 2016;18.

41. Law SC, Street S, Capini C, Ramnoruth S, Nel HJ, et al. T-cell autoreactivity to citrullinated autoantigenic peptides in rheumatoid arthritis patients carrying HLA-DRB1 shared epitope alleles. Arthritis Res Ther. 2012;14(3):R118.
42. Hill JA, Southwood S, Sette A, Cairns E**.** Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. J Immunol. 2003;171(2):538-41.
43. Scally SW, Petersen J, Law SC, Dudek NL, Nel HJ, et al. A molecular basis for the association of the HLA-DRB1 locus, citrullination, and rheumatoid arthritis. Journal of Experimental Medicine. 2013;210(12):2569-82.
44. James EA, Bui J, Berger D, Huston L**.** Tetramer-guided epitope mapping reveals broad, individualized repertoires of tetanus toxin-specific CD4+ T cells and suggests HLA-based differences in epitope recognition. Int Immunol. 2007;19(11):1291-301.
45. Snir O, Rieck M, Gebe JA, Yue BB, Rawlings CA, Nepom G, et al. Identification and functional characterization of T cells reactive to citrullinated vimentin in HLA-DRB1*0401-positive humanized mice and rheumatoid arthritis patients. Arthritis Rheum. 2011;63(10):2873-83.
46. Blachere NE, Parveen S, Fak J, Frank MO, Orange DE**.** Inflammatory but not apoptotic death of granulocytes citrullinates fibrinogen. Arthritis Res Ther. 2015;17:369.
47. Konig MF, Andrade F**.** A Critical Reappraisal of Neutrophil Extracellular Traps and NETosis Mimics Based on Differential Requirements for Protein Citrullination. Front Immunol. 2016;7:461.
48. Jones JD, O'Connor CD**.** Protein acetylation in prokaryotes. Proteomics. 2011;11(15):3012-22.

Chapter 4

Carbamylated autoantigens facilitate a breach in T cell tolerance

Jacqueline S. Dekkers[1], Jeroen N. Stoop[1], Peter A. van Veelen[2], Arnoud H. de Ru[2], George M.C. Janssen[2], Martin Hegen[3], Tom W. J. Huizinga[1], Leendert A. Trouw[1], René E.M. Toes[1]

1. Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands.
2. Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands.
3. Inflammation and Immunology Research Unit, Pfizer Worldwide Research and Development, USA.

ABSTRACT

Objectives

Posttranslational modifications (PTM) are thought to play a role in the breach of tolerance and onset of autoimmunity. In Rheumatoid arthritis (RA) autoantibodies directed against PTM proteins are implicated in disease pathogenesis, however why anti-modified protein responses are initiated in RA is still unknown. Here, we investigated whether posttranslational modification e.g. carbamylation of an antigen can result in the generation of neo-epitopes and subsequently induce a breach of tolerance towards self-antigens at a T cell level.

Methods

Mice were immunised with carbamylated or non-modified mouse Albumin (mAlb). Using pulsed DCs, T cell responses were studied by proliferation assays, IL-5 and IFN-γ ELISA. Digested carbamylated mouse albumin was analysed by Mass Spectrometry to identify carbamylated peptides.

Results

Studies in mice show that antigen specific T cell responses recognising carbamylated mAlb are induced by immunisation with carbamylated mAlb. Using mass spectrometry five carbamylated mouse albumin peptides were identified in digested carbamylated mouse albumin. Stimulation of spleen cells with carbamylated mouse albumin peptides induced a PTM specific T-cell response indicated by IFN-γ production.

Conclusions

These results indicate that carbamylation of self-proteins is sufficient for a breach of immunological tolerance at a T cell level. These results contribute to the concept that formation of anti-CarP antibodies occurs in a 'hapten'-like manner, suggesting that the help provided to anti-CarP antibody producing B cells can come from T cells directed against modified self-proteins or foreign-proteins.

Introduction

During normal physiological conditions, posttranslational protein modifications are important for biological function and critically influence protein structure and function. In Rheumatoid arthritis (RA) posttranslationally modified proteins (PTM) have been implicated in disease pathogenesis as different anti-modified protein antibody (AMPA) responses can be identified sera of RA patients (1-5). Why these autoimmune responses against PTM antigens are initiated in RA is currently unknown. It has been postulated that posttranslational modifications can play a role in the breach of tolerance towards self-antigens. A possible mechanism by which posttranslational modifications could break immune tolerance is that the modified self-antigen is not presented in the thymus, and therefore reactive T cells escape tolerance induction and migrate into the periphery. A second mechanism may be that antigen processing of proteins and peptides containing posttranslational modifications is different compared to the unmodified native counterparts allowing the presentation of otherwise 'cryptic' self-epitopes. Likewise, posttranslational modifications might affect the binding of the epitope to the major histocompatibility complex (MHC) class II molecules.

Besides citrullinated proteins, several other post-translational modifications can be targeted by antibodies in sera of RA patients. Anti-CarP antibodies are present in approximately 45% of RA patients and target proteins that are modified through an enzymatic modification named carbamylation. Carbamylation changes the charge of an amino acid (lysine has a positive charge, whereas homocitrulline has a neutral charge) and it can change the structure of a protein. This new feature could have implications for the binding of antibodies, recognition by T cell receptors and/or binding to MHC molecules. Given the observation that anti-CarP antibodies, in contrast to ACPA, do occur in both humans and mice, these autoantibodies enable us to study the etiology of AMPA responses in more detail.

We have previously shown that a breach in B cell tolerance could be readily induced upon immunization with carbamylated self- or foreign proteins leading to the formation of cross-reactive anti-CarP autoantibodies (6). The presence of different anti-CarP isotypes and subclasses upon immunization with carbamylated antigens and in sera of mice with collagen induced arthritis (7) is indicative of antigen-driven selection and T cell dependent antibody production against carbamylated autoantigens. It can be hypothesized that upon exposure to PTM self-proteins autoreactive T cell responses can be generated; however it is also conceivable that a T cell reaction against PTM foreign antigens provides help to self-reactive AMPA producing B cells. In this study we aimed to investigate whether posttranslational modification e.g. carbamylation of an antigen can result in the generation of neo-epitopes and subsequently induce a breach of tolerance towards self-antigens at a T cell level.

Methods

Carbamylation of mouse Albumin

For generating carbamylated mouse albumin (Ca-mAlb; EMD Millipore), proteins were diluted in PBS to a protein concentration of 10 mg/ml. The diluted protein was incubated for 12 hours at 37 °C with 1 M potassium cyanate (Sigma-Aldrich). After incubation the samples were extensively dialyzed against PBS.

Mice and immunizations

All animal experiments were approved by local regulatory authorities and conform national guidelines. C57BL/6 mice were purchased from Charles River. Mice were immunised when they were 8-10 week old mice. For aluminum hydroxide immunizations the mice received 2 intraperitoneal injections with a 1:1 mixture of antigen or peptide and alhydrogel (invivogen) containing 100 µg of antigen per immunization. Complete Freunds adjuvant (CFA) immunizations were done via injection at the tail base with 100 µg of antigen emulsified in complete Freunds adjuvant (CFA; Difco). Three weeks later mice received a subcutaneous boost with 100 µg of the same antigen in incomplete Freunds adjuvant (IFA; Sigma-Aldrich).

T cell assays

Spleen cells were harvested and cultured in RPMI supplemented with 8%FCS, penicillin, streptomycin, L-glutamin and 50 µ*M* 2-mercaptoethanol. Cells were stimulated with 20 µg/ml antigen and cultured for 4 days. After 4 days cytokine levels in the supernatant were determined by ELISA. For experiments with peptides, D1 dendritic cells (8) were pulsed for 24 hours with 20 µg/ml peptide, whole protein or protein digest in combination with 0.1µg/ml LPS. After D1 cell maturation cells were washed, irradiated with 3000 RAD and plated out with spleen cells in a 1:2 ratio. Cells were cultured four 4 days and after this cells were pulsed with ^{3}[H]-thymidine and incubated overnight at 37C and 5% CO_2. Cytokine levels in the supernatant were determined by IL-5 or IFN-γ cytokine ELISA.
Ca-mAlb was reduced with DTT and digested 2h at 37°C with either trypsin (enzyme protein ratio 1:20; pH 8.3), chymotrypsin (ratio 1:20; pH 8.3) or proteinase K (ratio 1:100; pH 11).

Protein digestion and mass spectrometry

Ca-mAlb was reduced with DTT and digested 2h at 37°C with either trypsin (enzyme protein ratio 1:20; pH 8.3), chymotrypsin (ratio 1:20; pH 8.3) or proteinase K (ratio 1:100; pH 11).
Chymotryptic peptides (from 500 µg Ca-mAlb) were separated on a 4.6 mm C18 reverse phase column equilibrated with 0.1% formic acid. Peptides were analyzed via on-line C18-nano-HPLC-MS with a system consisting of an Easy nLC 1000 gradient HPLC system (Thermo, Bremen, Germany), and a Q-Exactive mass spectrometer (Thermo). Fractions were injected

onto a homemade precolumn (100 µm × 15 mm; Reprosil-Pur C18-AQ 3 µm, Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (15 cm × 50 µm; Reprosil-Pur C18-AQ 3 um). The gradient was run from 0% to 30% solvent B (10/90/0.1 water/ACN/FA v/v/v) in 120 min. The nano-HPLC column was drawn to a tip of ~5 µm and acted as the electrospray needle of the MS source. The Q-Exactive mass spectrometer was operated in top10-mode. Parameters were set at resolution 70,000 at an AGC target value of 3,000,000, maximum fill time of 20 ms (full scan), and resolution 17,500 at an AGC target value of 100,000/maximum fill time of 60 ms for MS/MS at an intensity threshold of 17,400. Apex trigger was set to 1 to 5 seconds, and allowed charges were 1-6. In a post-analysis process, raw data were converted to peak lists using Proteome Discoverer 1.4 (Thermo). For peptide identification, MS/MS spectra were submitted to the mouse database using Mascot Version 2.2.04 (Matrix Science) with the following settings: 10 ppm and 20 mmu deviation for precursor and fragment masses, respectively; no enzyme was specified. All reported hits were assessed manually, and peptides with MASCOT scores <35 were generally discarded.

Statistical analysis

All statistical testing was performed using Prism 7 (GraphPad Software). Different groups of mice were compared using a Mann-Whitney U test or the Kruskal-Wallis test.

Results

Carbamylated self-antigens facilitate a breach in T cell tolerance.

Posttranslational modification of an antigen could potentially result in the generation of neo-epitopes and subsequently induce a breach of tolerance towards self-antigens at a T cell level. To study whether carbamylation of a self-antigen can have this effect, we immunised mice with either Ca-mouse albumin (Ca-mAlb) or non-modified-mouse albumin using a protein free adjuvant (aluminum hydroxide). Since aluminum hydroxide is known to be a Th-2 response skewing adjuvant in mice (9) IL-5 production was used as a outcome measure for T cell reactivity. Antigen specific INF-γ production by spleen cells from these mice could not be detected (data not shown). Spleen cells from Ca-mAlb immunized mice produced IL-5 after stimulation with Ca-mAlb *in vitro*, while mAlb stimulation did not induce IL-5 production (Figure 1A). To determine whether the IL-5 production was due to a Ca-mAlb specific effect, spleen cells of Ca-mAlb or mAlb immunised mice were restimulated *in vitro* with Ca-mAlb pulsed D1 dendritic cells (DCs). Spleen cells of mice previously immunised with Ca-mAlb gave a significant higher IL-5 production which suggests the loss of T cell tolerance towards a carbamylated self-antigen; however the induced T cell proliferation was less pronounced (Figure 1B).

When D1 DCs were pulsed with chymotrypsin or trypsin digested Ca-mAlb, IL-5 production was still induced by activation of spleen cells from Ca-mAlb immunised mice. Proteinase K digestion of Ca-mAlb results in very short peptides (less than 9 amino acids). After treatment with proteinase K, DCs pulsed with digested Ca-mAlb did not induce any IL-5 production (Figure 1C). Since, proteinase K digestion results in peptide fragments too short for MHCII presentation, this confirms that the observed IL-5 production is actually a T cell response to a protein antigen. To identify which epitope(s) of Ca-mAlb were recognized, chymotrypsin digested Ca-mAlb was fractioned based on hydrophobicity. Different fractions were then subsequently used to pulse DCs for T cell stimulation. DCs pulsed with fraction 2142941 induced an IL-5 response by spleen cells of Ca-mAlb immunised mice (Figure 1D). Fraction 2142941 was subsequently further analyzed by mass-spectometry and the most abundant Ca-peptides with a length of at least 9 amino acids were identified (Table 1). Only five identified peptides were actually derived from Ca-mAlb as other identified Ca-peptides had a different serological protein origin. We next aimed to test whether the identified Ca-mAlb peptides were capable of the induction of T cell stimulation and IL-5 production. To this end we immunised mice with Ca-mAlb and stimulated the spleen cells with either one of the five identified peptides pulsed DCs. After stimulation of the spleen cells we observed that all four immunised mice responded to peptide 7 E**k**LGEYGFQNAILVRY, the bold underlined non-capital k represents indicates the position of the homocitrulline residue (Figure 2A).

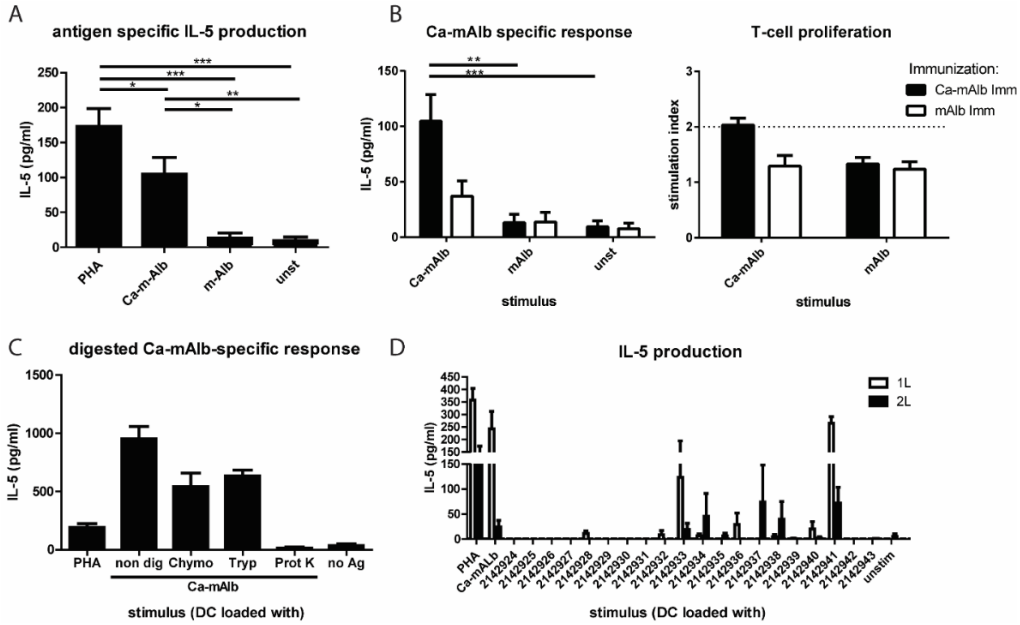


Figure 1. T cell activation and cytokine production after stimulation with carbamylated mouse Albumin. (A) Mice were immunised with carbamylated mouse albumin (Ca-mAlb) or native mouse albumin (mAlb) in aluminium hydroxide and boosted with the same antigen mixture. Spleen cells were *in vitro* stimulated with D1 cells pulsed with Ca-mAlb or mAlb. Phytohemagglutinin (PHA) mitogen was added to spleen cells as a positive control. IL-5 ELISA was performed as readout for T cell activation. (B) Spleen cells from mice immunised with Ca-mAlb (black bars) or mAlb (white bars) were *in vitro* stimulated with D1 cells pulsed with Ca-mAlb, mAlb or non-pulsed DCs. IL-5 ELISA was performed as readout for T cell activation (right panel) and cells were pulsed with ^{3}[H]-thymidine as a readout for T cell proliferation (left panel). The samples are pooled data from two independent experiments (n=10 mice per group). Statistical differences were determined by the Mann-Whitney U test or the Kruskal-Wallis test *p<0.05, **p<0.01, ***p<0.005. (C) Ca-mAlb digested by chymotrypsin, trypsin or proteinase K was used to pluse D1 cells. Spleen cells from mice immunised with Ca-mAlb were stimulated *in vitro* and an IL-5 ELISA was performed as readout (n=4 mice per group). (D) Chymotrypsin digested Ca-mAlb was fractioned based on hydrophobicity. D1 cells were pulsed with different fractions of mAlb and used for stimulation of spleen cells of Ca-mAlb immunised mice 1L (white bars) and 2L (black bars).

Peptide number	identified peptides	protein
1	DAGLTPNNLOPVAAEFYGSVEHPQTY	Serotransferin
2	MVOVLDAVRGSPAVDVAVOVF	Transthyretin OS
3	HWPQGPSTVDAAFSWDDOVY	Hemopexin OS
4	GVYVRATDLODWVQETMAON	Haptoglobin
5	RSVSELPIMHQDWLNGOEF	IgG1 chain contant region
6	ONPITSVDAAFRGPDSVF	Hemopexin
7	EOLGEYGFQNAILVRY	Serum albumin
8	DEHAOLVQEVTDFAOT	Serum albumin
9	VRQSPGOGLEWLGVIW	Ig heavy chain V region
10	DLGEQHFOGLVLIAF	Serum albumin
11	SQTFPNADFAEITOL	Serum albumin
12	VHLTDAEOAAVSGLW	Beta-globin
13	RGPDSVFLIOEDOVW	Hemopexin OS
14	YAEAODVFLGTF	Serum albumin

Table 1. List of homocitrullin conainting peptides in fraction 2142941 derived from carbamylated mouse Albumin. Chymotrypsin digested fractions of Carbamylated mouse Albumin were fractionated based on hydrophobicity. Fraction 2142941 was analysed by Mass Spectomery to identify homocitrulline containing peptides with a length of at least 9 amino acids.

In spleen cells stimulated with the other Ca-mAlb peptides similar T cell responses were observed although not all mice responded consistently. To determine whether the observed IL-5 response towards peptide 7 was homocitrulline specific we next stimulated spleen cells with DCs pulsed with peptide 7 that contained either a homocitrulline residue or a lysine residue at this particular position. Interestingly, the observed IL-5 response was in all 5 mice significantly higher towards the carbamylated version of peptide 7 (Figure 2B).

Finally, we aimed to determine whether immunization with carbamylated self-peptides could result in a breach homocitrulline specific T-cell tolerance. In a first experiment we immunised mice with peptide 7 containing a homocitrulline (Hcit) or a lysine (Lys) residue using aluminum hydroxide as an adjuvant. In this setting, low levels of IL-5 were detected in spleen cells cultures after stimulation with either the carbamylated or non-modified peptide 7 (Figure 3A and B). In a second experiment we therefore switched to Complete and Incomplete Freund's Adjuvant (CFA/IFA) to induce a Th-1 skewed immune response. Mice received two immunisations with Hcit or Lys peptide 7 emulsified in CFA/IFA. Antigen specific INF-γ production by spleen cells from these mice was subsequently measured as an outcome for T-cell reactivity. Spleen cells of mice immunised with Hcit peptide 7 (Figure 2C, left panel) responded with a significant higher IFN-γ production to the carbamylated

peptide compared to the control peptide. In mice immunised with Lys peptide7 no anti-carbamylated specific T cell responses were detected (Figure 2C, right panel). Together, these data show that posttranslational modification of a self-antigen can result in the generation of neo-epitopes and subsequently induce a breach of tolerance towards self-antigens at a T cell level.

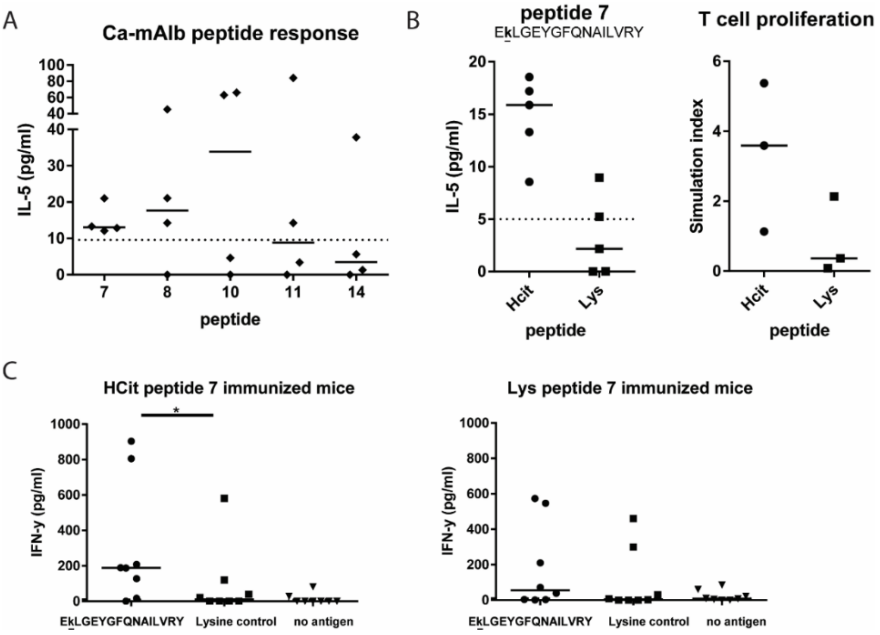


Figure 2. Homocitrulline specific T cell responses after stimulation with carbamylated peptide 7 (A) Mice were immunised with carbamylated mouse albumin (Ca-mAlb) in aluminium hydroxide and boosted with the same antigen mixture. Spleen cells were *in vitro* stimulated with D1 cells pulsed with different carbamylated peptides derived from Ca-mAlb. IL-5 ELISA was performed as readout for T cell activation. **(B)** Mice were immunised twice with Ca-mAlb in aluminium hydroxide. D1 cells were pulsed with a carbamylated or non-modified version of peptide 7 E**k**LGEYGFQNAILVRY, the bold underlined non-capital k represents indicates the position of the homocitrulline residue or the lysine residue. Spleen cells from Ca-mAlb immunised mice were subsequently *in vitro* stimulated with pulsed D1 cells and cultures were analysed for IL-5 production (n=5 mice per group, left panel) and T-cell proliferation (n=3 mice per group, right panel). **(C)** Mice were immunised with a carbamylated (left panel) or non-modified version of peptide 7 (right panel). Spleen cells were *in vitro* stimulated with D1 cells pulsed with carbamylated peptide 7, non-modified peptide 7 or with no antigen. IFN-γ ELISA was performed as readout for T cell activation. The samples are pooled data from two independent experiments (n=8 mice per group). Statistical differences were determined by the Kruskal-Wallis test, *p<0.05.

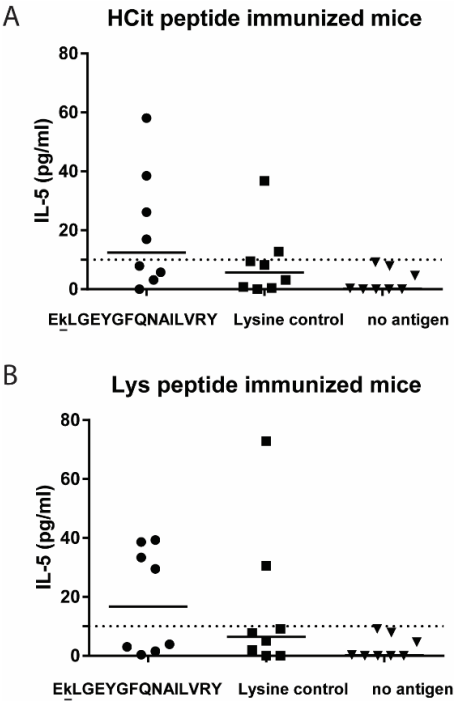


Figure 3. Low levels of IL-5 production after stimulation with carbamylated peptide 7 Mice were immunised with a carbamylated **(A)** or non-modified version of peptide 7 **(B)** using aluminum hydroxide. Spleen cells were *in vitro* stimulated with D1 cells pulsed with carbamylated peptide 7, non-modified peptide 7 or with no antigen. IL-5 ELISA was performed as readout for T cell activation. The samples are pooled data from two independent experiments (n=8 mice per group). Statistical differences were determined by the Kruskal-Wallis test.

Discussion

A key characteristic of one of the most common autoimmune diseases, rheumatoid arthritis, is the occurrence of autoantibodies against posttranslational modified proteins (10). We previously showed that carbamylation of both self- and foreign proteins is sufficient for a breach of immunological tolerance and the formation of anti-CarP antibodies. These findings show that posttranslational modification of self-proteins, in particular carbamylation, represents one way in which immune tolerance is bypassed both at a B cell and T cell level.

Carbamylated antigens have a different structure and charge, a feature which might have implications for antigen recognition and processing that can subsequently lead to a breach of tolerance towards self-antigens. Stimulation of spleen cells with Ca-antigen pulsed DCs led to the induction of a strong T cell response, cytokine production and proliferation, in contrast to stimulation with the non-modified version of the antigen. Protein digestion of Ca-mAlb with proteinase K resulted in short peptides that were too short for MHCII presentation and subsequent T cell activation. Stimulation of spleen cells with Ca-mAlb derived homocitrulline containing peptides identified by mass spectrometry resulted in a PTM specific T-cell response. Together these findings suggest that carbamylation of an autoantigen can result in the generation of neo-epitopes and subsequently induce a breach of tolerance towards self-antigens at a T cell level. Homocitrulline residues in a protein may function in a 'hapten-like manner' capable of eliciting a specific immune response once bound to a carrier-protein. Similar to posttranslational citrullination (11), carbamylation of antigens is, in itself, not specific for RA which demonstrated by the fact that anti-CarP antibodies have been described in different forms of autoimmune arthritis and other inflammatory diseases (12-14).

We consider it likely that anti-CarP immune responses can occur resulting from inflammatory conditions induced for example during infection, as it is conceivable that such conditions lead to carbamylation of self-proteins. Interestingly, carbamylated-albumin, which is able to induce a break of tolerance in mice was previously shown to be present in rheumatic joints of RA patients and antibodies reactive to carbamylated albumin have been found in approximately 40% of RA patients (6).

To summarize, we have shown that carbamylated proteins are able to trigger primary immune responses, including autoantibody production, T cell activation and cytokine production. Posttranslational modification of self-proteins by carbamylation is one way in which 'new' antigens are created for which immune tolerance does not exist. In the case of RA the formation of homocitrulline residues seems to promote tolerance loss and autoimmunity, it is unknown, however, whether immune responses to carbamylated proteins are directly pathogenic or a marker for inflammation.

References

1. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci U S A. 2011;108(42):17372-7.
2. Thiele GM, Duryee MJ, Anderson DR, Klassen LW, Mohring SM, Young KA, et al. Malondialdehyde-acetaldehyde adducts and anti-malondialdehyde-acetaldehyde antibodies in rheumatoid arthritis. Arthritis Rheumatol. 2015;67(3):645-55.
3. Juarez M, Bang H, Hammar F, Reimer U, Dyke B, Sahbudin I, et al. Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis. Ann Rheum Dis. 2016;75(6):1099-107.
4. Nienhuis RL, Mandema E**.** A New Serum Factor in Patients with Rheumatoid Arthritis; the Antiperinuclear Factor. Ann Rheum Dis. 1964;23:302-5.
5. Klareskog L, Ronnelid J, Lundberg K, Padyukov L, Alfredsson L**.** Immunity to citrullinated proteins in rheumatoid arthritis. Annu Rev Immunol. 2008;26:651-75.
6. Dekkers JS, Verheul MK, Stoop JN, Liu B, Ioan-Facsinay A, van Veelen PA, et al. Breach of autoreactive B cell tolerance by post-translationally modified proteins. Ann Rheum Dis. 2017;76(8):1449-57.
7. Stoop JN, Liu BS, Shi J, Jansen DT, Hegen M, Huizinga TW, et al. Antibodies specific for carbamylated proteins precede the onset of clinical symptoms in mice with collagen induced arthritis. PLoS One. 2014;9(7):e102163.
8. Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L, et al. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. J Exp Med. 1997;185(2):317-28.
9. Coffman RL, Sher A, Seder RA**.** Vaccine adjuvants: putting innate immunity to work. Immunity. 2010;33(4):492-503.
10. Willemze A, Trouw LA, Toes RE, Huizinga TW**.** The influence of ACPA status and characteristics on the course of RA. Nat Rev Rheumatol. 2012;8(3):144-52.
11. Baka Z, Gyorgy B, Geher P, Buzas EI, Falus A, Nagy G**.** Citrullination under physiological and pathological conditions. Joint Bone Spine. 2012;79(5):431-6.
12. Shi J, van Steenbergen HW, van Nies JA, Levarht EW, Huizinga TW, van der Helm-van Mil AH, et al. The specificity of anti-carbamylated protein antibodies for rheumatoid arthritis in a setting of early arthritis. Arthritis Res Ther. 2015;17:339.
13. Bergum B, Koro C, Delaleu N, Solheim M, Hellvard A, Binder V, et al. Antibodies against carbamylated proteins are present in primary Sjogren's syndrome and are associated with disease severity. Ann Rheum Dis. 2015.
14. Shi J, van Veelen PA, Mahler M, Janssen GM, Drijfhout JW, Huizinga TW, et al. Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies. Autoimmun Rev. 2014;13(3):225-30.

Chapter 5

Different classes of Anti-Modified Protein Antibodies are induced upon exposure to antigens expressing one type of modification

Annals of Rheumatic Diseases. 2019 Jul;78(7):908-916.

Adapted from: "Different classes of anti-modified protein antibodies are induced on exposure to antigens expressing only one type of modification."

J.S. Dekkers[¥,1], A.S.B. Kampstra[¥,1], M. Volkov[1], A.L. Dorjee[1], L. Hafkenscheid[1], A.C. Kempers[1], M. van Delft[1], T.Kissel[1], S. Reijm[1], G.M.C. Janssen[1], P.A. van Veelen[2], H. Bang[3], T.M.W. Huizinga[1], L.A. Trouw[5], D. van der Woude[1], R.E.M. Toes[1]

[¥] Contributed equally

1. Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands
2. Department of Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands
3. Research and development, Orgentec Diagnostika, Mainz, Germany.
4. Department of Immunohematology and Bloodtransfusion, Leids Universitair Medisch Centrum, Leiden, The Netherlands.

ABSTRACT

Objectives

Autoantibodies against post-translationally modified proteins (Anti-Modified Protein Antibodies or AMPA) are a hallmark of Rheumatoid Arthritis (RA). A variety of classes of AMPAs against different modifications on proteins, such as citrullination, carbamylation and acetylation, have now been described in RA. At present, the origin or mutual relationship of AMPAs is poorly understood. Here, we aimed to study the origin of AMPA-responses by postulating that the AMPA-response shares a common "background" that evolves into different classes of AMPAs.

Methods

Mice were immunized with acetylated-, carbamylated- or non-modified ovalbumin and analyzed for AMPA-responses. In addition, serum reactivity towards modified antigens was determined for RA patients.

Results

Immunisation of mice with carbamylated proteins induced an antibody response not only recognizing carbamylated proteins, but also acetylated proteins. Similarly, immunization with acetylated proteins led to the formation of (autoreactive) AMPAs against other modifications as well. Analysis of antibodies purified from blood of RA patients using citrullinated antigens revealed that these antibodies, besides being citrulline-reactive, can also display reactivity to acetylated and carbamylated peptides. Similarly, affinity-purified anti-carbamylated protein antibodies showed cross-reactivity against all three post-translational modifications tested.

Conclusions

Our data show that different AMPA-responses can emerge from exposure to only a single type of modified protein. These findings indicate that different AMPA-responses can originate from a common B cell response that diversifies into multiple distinct AMPA-responses and explain the presence of multiple AMPAs in RA, one of the hallmarks of disease.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease affecting the synovial joints. The disease is characterized by the presence of autoantibodies, recognizing self-proteins. The most extensively studied antibodies are Rheumatoid Factor (RF), recognizing the Fc-part of IgG molecules, and Anti- Citrullinated-Protein Antibodies (ACPA), antibodies that recognize a particular Post-Translational Modification (PTM) on proteins. Due to the specificity of these autoantibodies for RA, sero-status of these autoantibodies has been included in the 2010 ACR/EULAR classification criteria for RA (1).

ACPAs have been shown to recognize citrullinated proteins, a PTM resulting from the deimidation of arginine within proteins. The process of deimination is mediated by PeptidylArginine Deiminases (PAD), enzymes present in all human cells and involved in several cellular processes (reviewed in (2)). Both polyclonal and monoclonal ACPA can display an extensive citrulline-dependent cross-reactivity towards multiple citrullinated peptides and proteins (3). Interestingly, the citrullinated epitope-recognition profile expands before clinical onset of disease, possibly as a consequence of the activation of new ACPA-expressing B cells and/or progressive somatic hypermutation of individual B cell clones (4-8). Besides autoantibodies targeting citrullinated antigens, other post-translationally modified proteins have been found to be recognized by autoantibodies, in particular carbamylated and acetylated proteins (9). Both carbamylation and acetylation are conversions of lysine. Carbamylation is a chemical process mediated by cyanate, a compound increased during inflammation (10). The resulting homocitrulline resembles citrulline, but contains an additional methylene group. Acetylation can occur through intracellular acetyltransferases, a process that can be mimicked chemically in vitro. Anti-Carbamylated protein (anti-CarP) antibodies are present in approximately 45% of early RA patients (11). These antibodies can be cross-reactive to citrullinated antigens, but can also display a more restricted recognition profile directed against carbamylated proteins only. Indeed, 10-20% of ACPA-negative RA patients are positive for anti-CarP antibodies, indicating that these antibodies represent a different class of Anti-Modified Protein Antibodies (AMPA) (11). Anti-Acetylated Protein Antibodies (AAPAs) have been reported to be present in approximately 40% of RA patients(12). The presence of these antibodies was mainly found in ACPA-positive RA, although also some ACPA-negative RA patients were positive for AAPA. Inhibition experiments showed limited cross-reactivity between anti-acetylated, anti-carbamylated and anti-citrullinated peptide antibodies, indicating that also AAPA represent another class of AMPA (12). These previous observations are interesting as they indicate that AMPA-reactivity, due to their combined appearance in RA, has a commonality that is currently not understood. Here, we studied the possibility that the AMPA-response originates from a common event by analyzing whether exposure to one particular class of modified proteins can generate different AMPA-responses.

Materials and Methods

Proteins and modifications

Mouse albumin was purchased from Merck Millipore (Cat# 126674), human fibrinogen and chicken ovalbumin (OVA) were purchased from Sigma Aldrich (Cat# F4883 and Cat# A5503 respectively). Carbamylation of proteins was achieved by incubating the proteins with potassium cyanate (Cat#215074, Sigma Aldrich) as has been described before (11). In short, OVA and mouse albumin were incubated overnight at 37°C in an end concentration of 1M potassium cyanate at a protein concentration ranging between 1 and 5mg/mL. Human fibrinogen was incubated in 0.5M potassium cyanate for 3 days at 4°C. All proteins were subsequently extensively dialyzed in PBS for 3 days.

Acetylation was performed as previously described (13). In short, proteins were diluted to a concentration of 1mg/mL in 0.1M Na2CO3. Per 20mL of protein solution, 100uL of acetic anhydride was added and subsequently 400uL of pyridine. Proteins were incubated at 30°C for 5 hours or overnight whilst shaking. After incubation, the acetylation reaction was stopped by adding 400uL (per 20mL solution) of 1M Tris. Acetylated proteins were purified by exchanging the buffer for PBS through Zeba Spin Desalting columns (Thermo Scientific).

Citrullination of OVA and fibrinogen was performed by incubation of the proteins with PeptidylArginine Deiminase (PAD) 4 enzyme (Cat# 1584, Sigma Aldrich) in the presence of 0.1M Tris-HCl (pH 7.6) and 0.15M CaCl2. For OVA, 3 units of PAD were added per mg of protein for the citrullination process whereas for fibrinogen 5U PAD per mg protein was used. Both proteins are incubated overnight at 53°C. Modifications were validated by ELISA.

ELISA modified antigens

Modification of fibrinogen and OVA were validated by ELISAs using commercial polyclonal rabbit anti-carbamyl-lysine antibodies (Cat# STA-078, Cell Biolabs) and commercial polyclonal rabbit antiacetylated-lysine antibodies (Cat# ADI-KAP-TF120-E, Enzo Lifesciences), or our human ACPA monoclonal antibody as described in (14). In short, proteins were coated at a concentration of 10µg/mL (in 0.1M carbonatebicarbonate buffer, pH 9.6) on Nunc Maxisorp plates (Cat# 430341, Thermofisher Scientific) and incubated overnight at 4°C. Wells were blocked with PBS + 2% BSA to inhibit unspecific antibody binding to the plastic for 4 hours at 4°C before incubating the plates with the anti-carbamyl-lysine antibodies, anti-acetylated-lysine antibodies or the ACPA monoclonal (diluted in RIA buffer containing 10mM TRIS (pH 7.6), 350mM NaCl, 1% TritonX, 0.5% Nadeoxycholate and 0.1% SDS) overnight at 4°C. Binding of the antibodies was detected by a goat-antirabbit Horse RadishPeroxidase (HRP)-conjugated antibody (for the rabbit polyclonal antibodies) (#P0448, DAKO) or a rabbit-anti-human-IgG HRP-conjugated antibody (for the human ACPA monoclonal) (Cat# P0214, DAKO) (4hrs at 4°C or 2hrs at RT). HRP content was visualized by

incubation with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) with 1:2000 H2O2.

Mass spectrometry

Mass spectrometry for MS analysis, modified proteins and their non-modified counterparts were subjected to 4-12% PAGE (NuPAGE Bis-Tris Precast Gel, Life Technologies). Bands were cut from the gel, and the proteins subjected to reduction with dithiothreitol, alkylation with iodoacetamide and in-gel trypsin digestion using Proteineer DP digestion robot (Bruker). Tryptic peptides were extracted from the gel slices, lyophilized, dissolved in 95/3/0.1 v/v/v water/acetonitril/formic acid and subsequently analyzed by on-line C18 nanoHPLC MS/MS with a system consisting of an Easy nLC 1000 gradient HPLC system (Thermo, Bremen, Germany), and a LUMOS mass spectrometer (Thermo).

Fractions were injected onto a homemade precolumn (100 µm × 15 mm; Reprosil-Pur C18-AQ 3 µm, Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (15 cm × 50 µm; Reprosil-Pur C18-AQ 3 um). The gradient was run from 10% to 40% solvent B (20/80/0.1 water/acetonitrile/formic acid (FA) v/v/v) in 20 min. The nano-HPLC column was drawn to a tip of ∼5 µm, and acted as the electrospray needle of the MS source. The LUMOS mass spectrometer was operated in data-dependent MS/MS (top-10 mode) with collision energy at 32 V and recording of the MS2 spectrum in the orbitrap. In the master scan (MS1) the resolution was 120,000, the scan range 400-1500, at an AGC target of 400,000 at maximum fill time of 50 ms. Dynamic exclusion after n=1 with exclusion duration of 10 s. Charge states 2-5 were included. For MS2 precursors were isolated with the quadrupole with an isolation width of 1.2 Da. HCD collision energy was set to 32 V. First mass was set to 110 Da. The MS2 scan resolution was 30,000 with an AGC target of 50,000 at maximum fill time of 60 ms. In a post-analysis process, raw data were first converted to peak lists using Proteome Discoverer version 2.1 (Thermo Electron), and then submitted to the Uniprot database (452772 entries), using Mascot v. 2.2.04 (www.matrixscience.com) for protein identification. Mascot searches were with 10 ppm and 0.02 Da deviation for precursor and fragment mass, respectively, and trypsin as enzyme. Up to two missed cleavages were allowed, and carbamidomethyl on Cys was set as a fixed modification. Methionine oxidation, carbamylation (Lys) and acetylation (Lys) were set as variable modification.

Protein modifications were finally compared using Scaffold software version 4.7.5 (www.proteomesoftware.com). The interpretation of MS2 spectra of modified peptides were also manually judged. Abundances were estimated using Proteome Discoverer workflow.

Mouse immunizations

8-10 week-old female C57BL6/J mice were purchased from Charles River. Mice received two injections i.p. with antigen (100ug) emulsified in Alhydrogel (Cat# vac-alu-250, Invivogen) in a 1:1 ratio. Animal experiments were approved by the local Ethical Committee for Animal Experimentation and performed conform national guidelines. All immunized mice were healthy and showed no signs of autoimmunity throughout the experiment.

Detection of Anti-Modified-Protein Antibodies

For the detection of AMPAs in mice, the following Enzyme-Linked ImmunoSorbent Assay (ELISA) was performed: Modified proteins and their unmodified counterparts were coated at a concentration of 10ug/mL in 0.1M carbonate-bicarbonate buffer (pH 9.6) overnight on Nunc Maxisorp plates (Thermo Scientific). The plates were blocked with PBS + 1% BSA. The mouse sera were diluted in RIA buffer (10mM TRIS (pH 7.6), 350mM NaCl, 1% TritonX, 0.5% Sodiumdeoxycholate, 0.1% SDS) and incubated overnight. Binding of mouse IgG was detected with HorseRadish Peroxidase (HRP)-conjugated goat-anti-mouse IgG1 (Cat# 1070-05, Southern Biotech) and subsequently visualized with ABTS. Washing steps were performed between each incubation with PBS + Tween20. All incubations, aside from the incubations with goat-anti-mouse IgG1 and ABTS, were performed at 4°C, the final two steps were performed at room temperature. Arbitrary units were calculated using a standard serum serial dilution. For the inhibition experiments, the sera were pre-incubated with 0 – 0.2mg/mL protein for 1 hour before transferring them to the ELISA plate. For avidity studies, wells were incubated for 15 minutes at RT with sodium thiocyanate (0 – 5M) after sera incubation. After the 15 minutes of incubation, the standard protocol was proceeded. The Relative Avidity Index (RAI) was calculated as previously been described in been described in (15):

$$RAI = \frac{antibodies\ remaining\ at\ 1M\ SCN\ (\frac{AU}{mL})}{antibodies\ bound\ at\ 0M\ SCN\ (\frac{AU}{mL})} \text{ x } 100$$

Reactivity of purified ACPA and Anti-CarP antibodies, obtained from sera and synovial fluid of RA patients, was measured using modified vimentin peptides (plates and reagents were kindly provided by Orgentec), according to the protocol previously described (12). In short, sera and purified antibodies were diluted 200ug/mL and 2,5ug/mL respectively for ACPA and 100ug/mL and 2.5ug/mL respectively for anti-CarP in diluent and incubated for 30'. Subsequently enzyme conjugate was added for 15' and reactivity was detected by TMB substrate. All steps were performed at room temperature. In addition, purified ACPA and anti-CarP-antibodies were tested on CCP2 and Ca-FCS respectively according to protocols previously described (11, 16).

IgG-AMPA purification

Specific AMPAs are isolated as has been previously described in (17). In short, plasma or serum samples and SF were acquired from patients. Synovial fluid was treated with hyaluronidase (derived from bovine testes type IV; Cat#: H3884, Sigma Aldrich) for 30 minutes at room temperature, before centrifugation at 3000 RPM for 10 minutes. The plasma, serum and SF samples were subsequently filtered (0.2µM filters, Millipore) before purifying AMPA with protein affinity chromatography (ÄKTA, GE Healthcare). Purification was performed using HiTrap streptavidin HP 1ml columns (GE-Healthcare) coupled with biotinylated CCP2-peptides (obtained from J.W. Drijfhout, IHB LUMC) for the isolation of ACPA (17) or in-house prepared biotinylated (Ca-)FCS for the isolation of anti-CarP antibodies. PTM-specificity was controlled by attaching a control column coated with the native version (CCP2 arginine or FCS) before the column coated with the modified version (CCP2 citrulline or Ca-FCS). Antibodies were eluted using 0.1M glycine hydrogen chloride (HCl) pH 2.5 and neutralized with 2M Tris. ACPA-IgG1,2,4 was subsequently purified from ACPA with Prot A and Prot G 156 HiTrap-columns.

FCS was biotinylated using EZ-link Sulfo-NHS-biotin (Pierce) incubated for ~24h on ice at 4°C at low pH to ensure N-terminal biotinylation (end concentration 20mg/ml FCS and 10mg/ml biotin solution), with subsequent extensive dialyzing. Part of the biotinylated FCS was carbamylated using 1M KOCN and the other part (native control) not. Both were incubated for ~12h at 37°C and extensively dialyzed afterwards.

Statistics

Statistical tests were performed with Prism7 (Graphpad). Significance of AMPA reactivity on proteins was tested with paired t-test. Differences in titer and avidity were tested with Mann-Whitney U tests. Correlations were assessed with Spearman. A p-value of <0.05 was considered significant.

Results

Cross-reactive AMPA are induced upon vaccination with one defined modified antigen.

To analyze whether AMPA can be induced upon immunization with an antigen with one defined modification and to determine whether these AMPA also recognize other classes of PTMs, we immunized mice with either non-modified, carbamylated, citrullinated or acetylated Ovalbumin (OVA). The presence of either homocitrulline as a result of carbamylation or acetylated lysine as a consequence of acetylation was confirmed by mass spectrometry (raw MS data are available online at the ProteomeXchange) and commercially available antibodies against either carbamylated or acetylated lysines in ELISA (Figure 1A). Non-modified OVA was found to be acetylated, but not carbamylated, at the N-terminus by mass-spectrometry and therefore the latter antigen was included in all immunization experiments as additional specificity control. To discriminate between reactivity against the PTM and protein-backbone used for immunization, we used modified fibrinogen instead of modified OVA as read-out. In doing so, antibodies recognizing OVA were not interfering with the detection of AMPA (18). Fibrinogen was not recognized by commercially antibodies against either carbamylated or acetylated lysine, indicating the absence of PTMs in non-modified fibrinogen (figure 1B). To control for possible baseline-reactivity towards modified proteins, sera from non-immunized mice were taken along in the ELISA experiments. Indeed, no reactivity was observed to non-modified fibrinogen or its modified counterparts in naïve animals, indicating that without immunizations, AMPA-responses are not present (Figure 2A) (18, 19). Likewise, although a strong reaction against OVA was noted (data not shown), indicating proper immunization, mice immunized with unmodified OVA did not harbor a reaction against CaFib, AcFib or CitFib (Figure 2B) (18). These results indicate that neither non- modified OVA or the adjuvant used is driving AMPA production. Despite reported ACPA presence in murine models after different immunization strategies (20-22), we were unable to detect reactivity towards citrullinated fibrinogen using our standard vaccination strategy (Figure 2C). Antibody reactivity was additionally tested towards modified Myelin Basic Protein (MBP), showing no reactivity towards the citrullinated form (Figure 3). Furthermore, mice immunized with carbamylated OVA (Ca-OVA) displayed a strong reactivity towards carbamylated fibrinogen (Ca-Fib), but not non-modified fibrinogen as determined in ELISA (Figure 2D). These data indicate that anti-CarP-antibodies are induced by immunization with Ca-OVA, but not by non-modified or citrullinated OVA. Remarkably, the sera of mice immunized with Ca-OVA also reacted to Ac-Fib and to some extend to citrullinated fibrinogen (Figure 2D). This reactivity was further validated by ELISA using modified MBP (Figure 3). These data are intriguing as they indicate that antibody responses induced by carbamylated antigens are able to recognize multiple modifications,

pointing to the presence of cross-reactive antibodies against one class of modified proteins that is induced by exposure to another class of modified proteins.

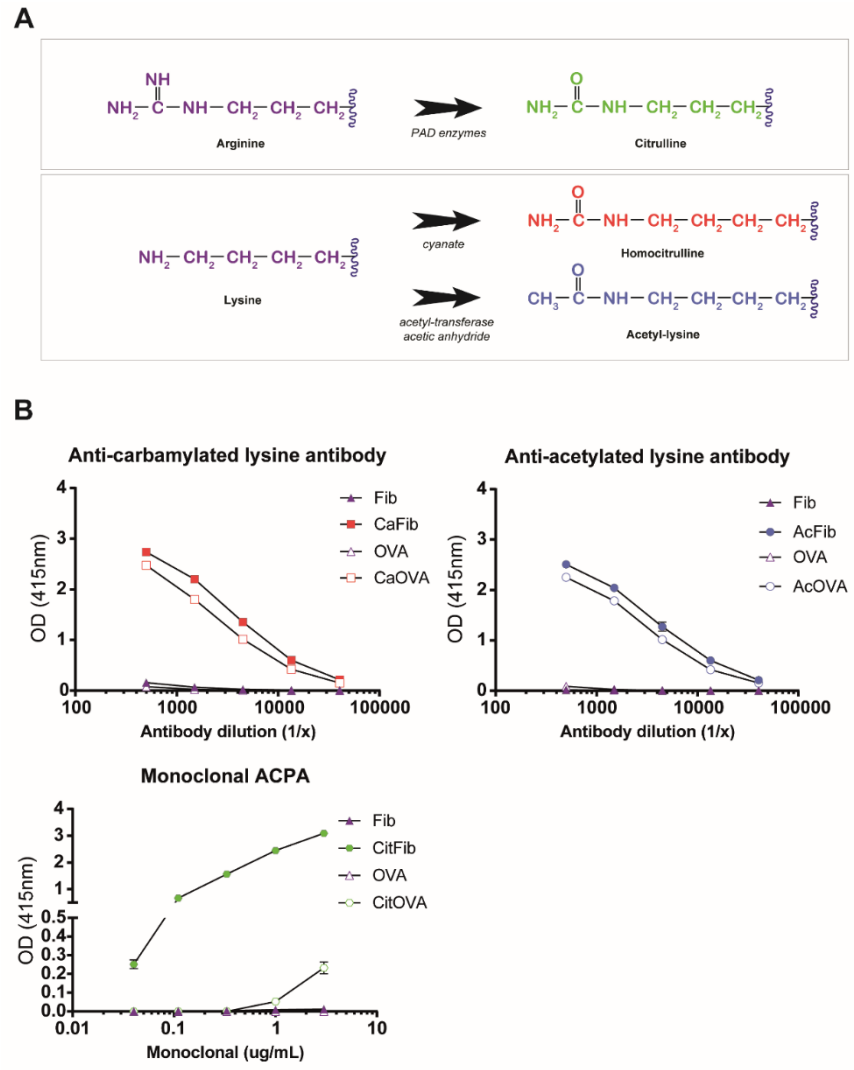


Figure 1. Structural overview of the posttranslational protein modifications. Schematic view of the amino acid structures of arginine and lysine, and their conversions towards citrulline, homocitrulline and acetylated lysine (A). ELISA with commercial polyclonal anti-acetylated-lysine antibodies, polyclonal anti-carbamylated-lysine antibodies or monoclonal ACPA to test modified proteins for the presence of post-translational modifications (B). OVA, ovalbumin; Fib, fibrinogen; Ca, carbamylated; Cit, citrullinated; Ac, acetylated; OD, optical density; PAD, peptidylarginine deiminase; ACPA, anti-citrullinated-protein antibodies; ug/mL, microgram per milliliter.

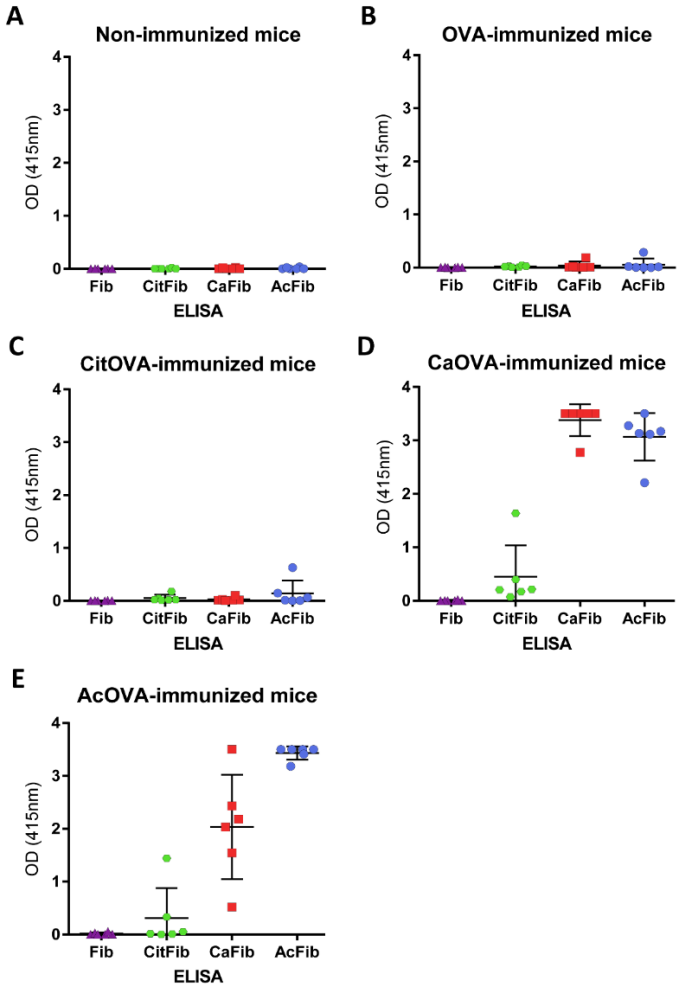


Figure 2. Immunization with CaOVA or AcOVA induces antibody responses towards modified fibrinogen. Immunization with Ca-OVA or Ac-OVA induces antibody responses towards modified fibrinogen. Antibody reactivity towards modified fibrinogen in sera derived from non-immunized (A), OVA-immunized (B), Cit-OVA-immunized (C), Ca-OVA-immunized (D) or Ac-OVA-immunized (E) mice was measured by ELISA. Reactivity is depicted with OD values measured at 415nm. For all groups, n = 6. Representative data from two experiments are shown. ** = p-value of <0.005 ;**** = p-value of <0.0001. OVA, ovalbumin; Cit, citrullinated; Ca, carbamylated; Ac, acetylated; Fib, fibrinogen; OD, optical density.

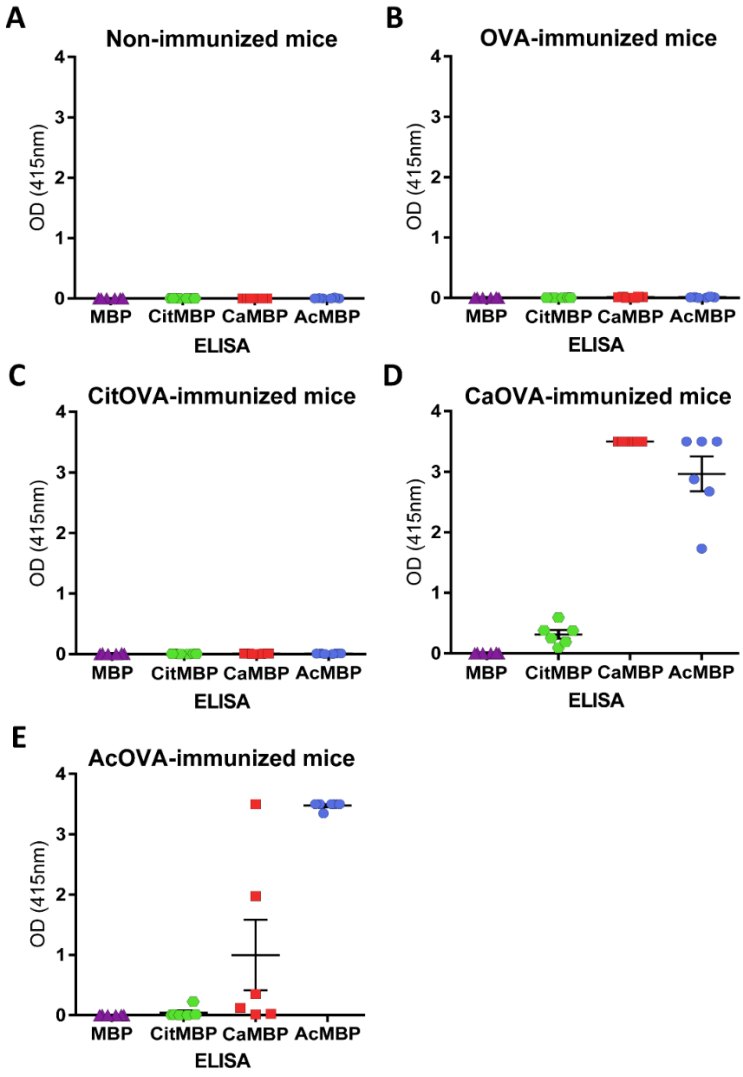


Figure 3. Immunization with CaOVA or AcOVA induces antibody responses towards modified MBP. Antibody reactivity towards modified MBP in sera derived from non-immunized (A), OVA-immunized (B), CitOVA-immunized (C), CaOVA-immunized (D) or AcOVA-immunized (E) mice was measured by ELISA. Reactivity is depicted with OD values measured at 415nm. For all groups, n = 6. Representative data from two experiments is shown. OVA, ovalbumin; Cit, citrullinated; Ca, carbamylated; Ac, acetylated; MBP, myelin basic protein; OD, optical density.

Next, we wished to determine whether cross-reactive anti-Ac-Fib antibodies could also be induced by immunization with acetylated OVA. We observed that reactivity against Ac-Fib was readily generated by immunization with Ac-OVA as expected, but reactivity towards Ca-Fib was also clearly detectable (Figure 2E). On the other hand, reactivity towards citrullinated fibrinogen was only moderately apparent, which could not be validated using cit-MBP as antigen (Figure 3). These results suggest that immunization with acetylated OVA induces antibodies cross-reactive to homocitrulline.

To further investigate the cross-reactive nature of the AMPA-responses generated, we next analyzed the level and titer of anti-Ca-Fib and Ac-Fib antibodies in mice immunized with respectively Ca-OVA or Ac-OVA. A strong correlation was noted in reactivity towards Ac-Fib and Ca-Fib in mice immunized with Ca-OVA (Figure 4A). Although all, but one, mice mounted a high level of antibodies recognizing Ac-Fib, some mice displayed a high level of reactivity towards Ca-Fib, whereas others showed a lower level of Ca-Fib-reactivity (Figure 4B). These findings were further substantiated by determining the titer of anti-Ca-Fib- and anti-Ac-Fib-antibodies through dilution of sera from immunized animals. Similar antibody titers were observed towards Ac-Fib and Ca-Fib in mice immunized with Ca-OVA, whereas the titer of antibodies recognizing Ac-Fib was considerable higher than the antibody-titer against Ca-Fib in Ac-OVA-immunized mice (Figure 5A).

These data indicate that the antibody response induced in Ca-OVA-immunized mice displays similar reactivity towards both Ca-Fib and Ac-Fib, suggesting that the antibody response induced in this setting is possibly cross-reactive. Likewise, these data also suggest that this reactivity is different in Ac-OVA-immunized mice as the antibody titer against acetylated antigens is considerably higher compared to the antibody-titer directed against carbamylated proteins, and hence that not all AAPA cross-react to Ca-Fib. Thus, together these data imply that cross-reactive antibodies to acetylated and carbamylated antigens are induced upon vaccination with only carbamylated- or acetylated antigens, though the nature of these responses might differ from each other.

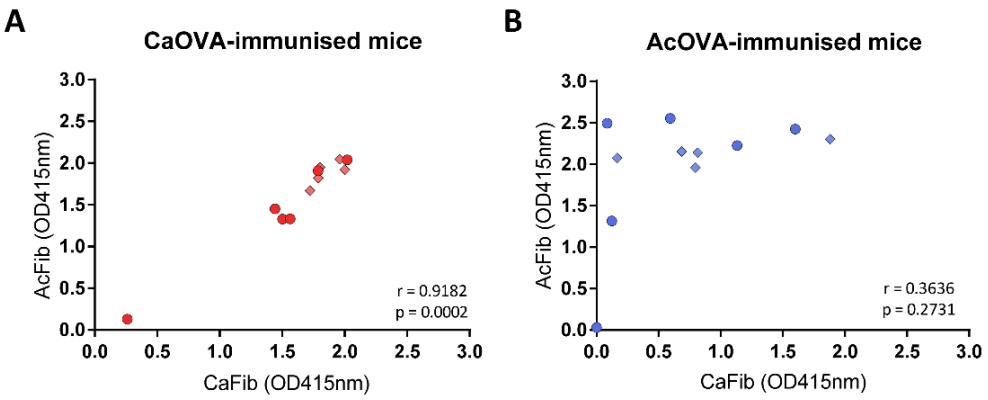


Figure 4. Correlation between CaFib- and AcFib-reactivity for CaOVA- and AcOVA-immunized mice. Reactivity towards CaFib and AcFib in sera from CaOVA- (A) and AcOVA-immunized (B) mice was measured with ELISA and correlated to each other. Data shown for two separate immunization experiments (diamonds (n=5) and circles (n=6)). Correlations are analyzed with Spearman, $p < 0,05$ depicts significance. OVA, ovalbumin; Ca, carbamylated; Ac, acetylated; Fib, fibrinogen; OD, optical density.

Cross-reactive antibody responses harbor different PTM recognition profiles, including differences in avidity.

To examine the AMPA-response in more detail, the avidity of the anti-CarP- and AAPA-response was determined by means of elution ELISAs using chaotropic salt (23, 24). Our results indicate that in Ca- OVA-immunized mice, the avidity of the antibody response to Ca-Fib is higher than the response to Ac-Fib showing a Relative Avidity Index (RAI) at 1M SCN of 58.5 vs 32.8 (Figure 5B). Furthermore, for the Ac-OVA-immunized mice, the avidity of the response towards Ac-Fib was somewhat higher as compared to the response towards Ca-Fib (RAI at 1M of 47.1 vs 31.5 respectively) (Figure 5B). Thus, these data indicate that the avidity of the AMPA-response is highest towards the respective modification used for immunization. The data presented in figure 5A indicate that immunization of mice with acetylated proteins induces an antibody response that is only partly cross-reactive to carbamylated antigens, whereas most antibodies induced by immunization with carbamylated antigens recognized both acetylated- and carbamylated antigens. These data imply that inhibition studies using acetylated proteins and sera from Ca-OVA-immunized mice (highly cross-reactive) would show good inhibition of reactivity towards carbamylated and acetylated antigens. In contrast, inhibition of reactivity towards acetylated antigens by carbamylated proteins of sera from Ac-OVA-immunized mice is predicted to be modest as most antibodies will not be cross-reactive. To test and confirm these notions, the binding

capacity towards Ca-Fib or Ac-Fib was analyzed after pre-incubation with increasing concentrations of modified fibrinogen. Indeed, for the Ca-OVA-immunized mice, the antibody reactivity towards either Ca-Fib or Ac-Fib could be inhibited by incubating the sera with Ca-Fib or Ac-Fib (Figure 6A and 6B). In contrast, whereas Ca-Fib-reactivity by Ac-OVA-immunized mice was blocked by incubation with Ca-Fib or Ac-Fib (Figure 6C), Ac-Fib reactivity could only be inhibited by competing with Ac-Fib (Figure 6D). These data further verify that antibodies generated by Ca-OVA-immunization are highly cross-reactive in nature, whereas for the AMPA induced by Ac-OVA-immunization, only a part of the antibodies are cross-reactive towards both modifications.

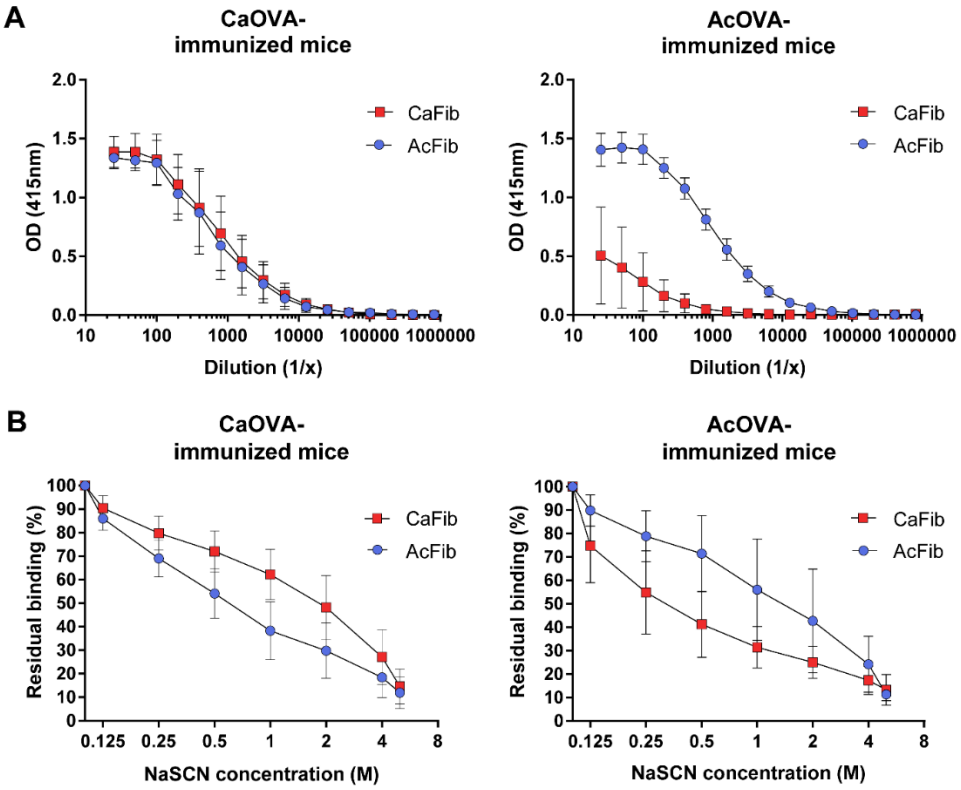


Figure 5. Antibody titers and avidity in sera of CaOVA- and AcOVA-immunized mice. Antibody titers as measured by ELISA on CaFib and AcFib for CaOVA- and AcOVA-immunized mice (A). IC50 depicts the dilution at which half of the highest reactivity is still visible. Representative data from two experiments is shown. (B) Avidity shown as residual antibody binding in the presence of different concentrations of NaSCN. Representative data from two immunization experiments is shown. Ca, carbamylation; Ac, acetylation; OVA, ovalbumin; Fib, fibrinogen; IC50, inhibitory concentration at 50%; OD, optical density; NaSCN, Sodium Thiocyanide; M, molar.

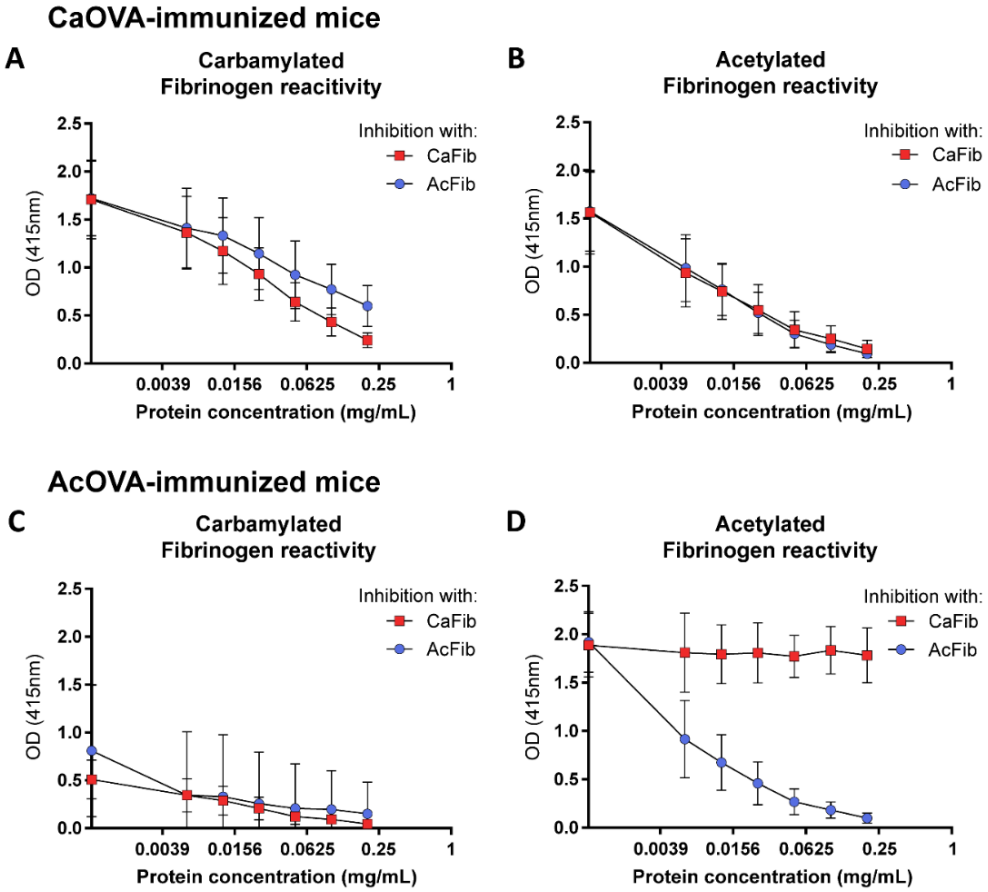


Figure 6. Inhibition of antibody binding by pre-incubation of mouse sera with modified fibrinogen. Cross-reactivity of antibodies is studied by assessment of the inhibitory capacity of pre-incubating sera with modified fibrinogen. Sera from CaOVA-immunized mice was pre-incubated with varying concentrations of modified fibrinogen before testing the antibody reactivity on CaFib (A) or AcFib (B). Sera from AcOVA-immunized mice was pre-incubated with varying concentrations of modified fibrinogen before testing the antibody reactivity on CaFib (C) or AcFib (D). Results show representative data of two experiments. OVA, ovalbumin; Fib, fibrinogen; Ca, carbamylated; Ac, acetylated; OD, optical density; mg/mL, milligram per milliliter.

Immunization with modified foreign-antigen is able to induce a breach of tolerance towards different classes of modified self-antigens.

The data presented above are important as they indicate that exposure to a particular class of modified proteins can induce an immune response against another class of modified proteins as well. However, these experiments did not address the question whether the antibodies induced are autoreactive, i.e. able to recognize modified self-proteins. Therefore, we next wished to investigate whether the AMPA-responses induced by exposure to foreign modified proteins bind to modified self-proteins as well. To this end, sera of immunized mice were tested for reactivity towards modified mouse albumin (mAlb) as model self-protein. Non-immunized mice or mice immunized with non-modified OVA did not react towards native or modified mAlb (Figure 7A). In contrast, sera from Ca-OVA-immunized mice reacted to both Ac-mAlb and Ca-mAlb (Figure 7A). Likewise, sera from Ac-OVA-immunized mice recognized both classes of modified mAlb as well, albeit that the reactivity towards Ca-mAlb was relatively low in concordance with the data depicted in Figure 2. Additionally, when the reactivity towards Ac-mAlb and Ca-mAlb was correlated for both immunized groups, a similar difference in correlation was apparent as is shown in figure 2 (Figure 7B). Together, these results indicate that exposure to modified foreign proteins is capable of inducing a breach of tolerance towards self-antigens carrying different classes of modifications.

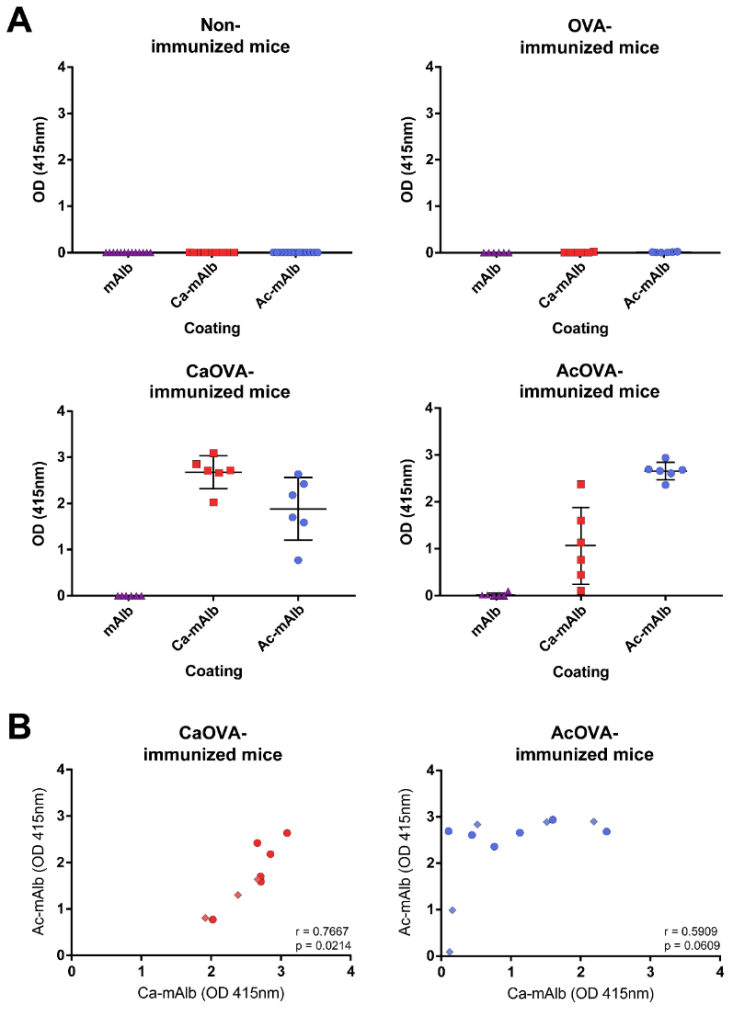
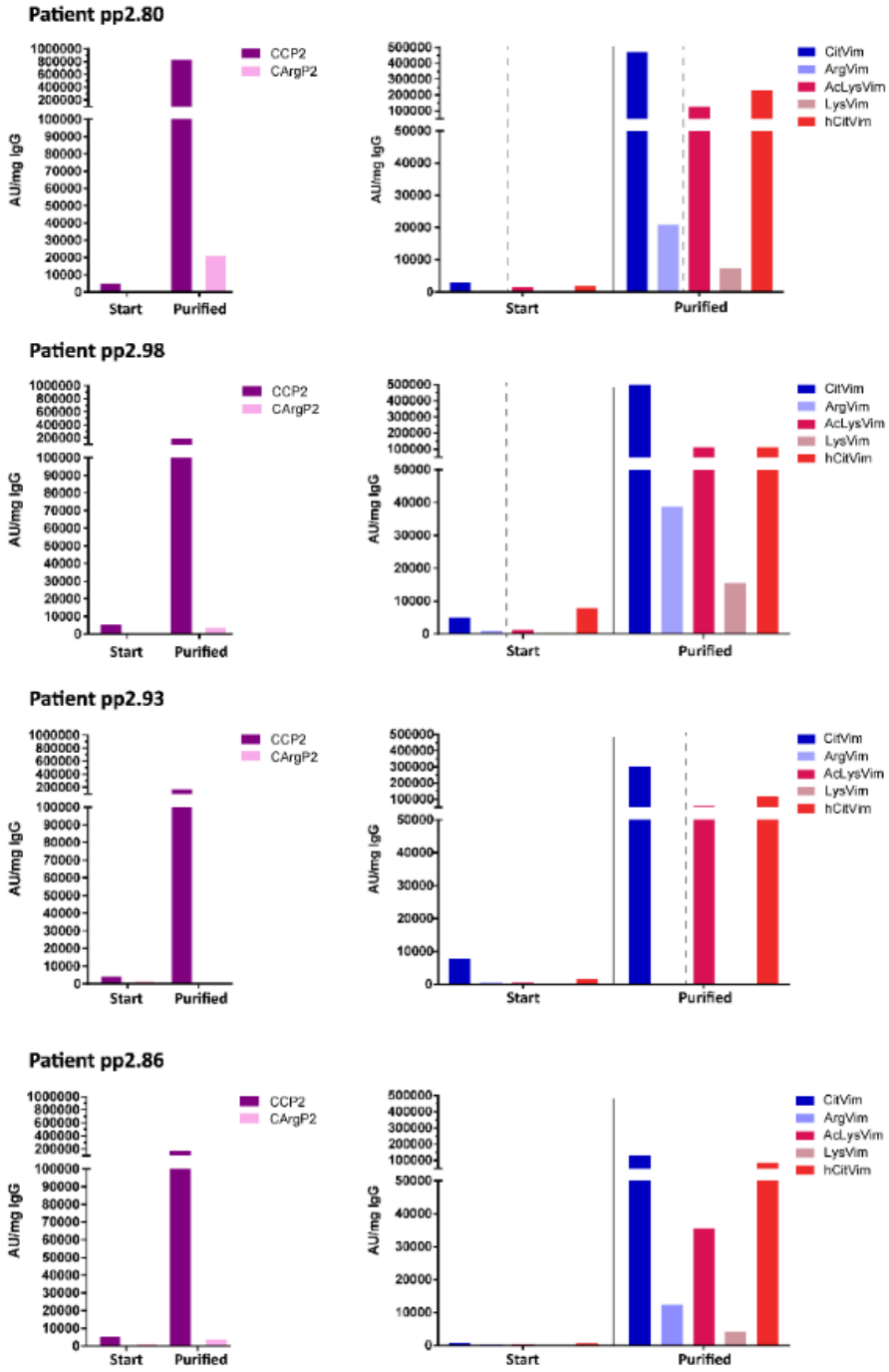


Figure 7. Break of tolerance towards modified self-proteins in CaOVA- and AcOVA-immunized mice. Reactivity in towards carbamylated and acetylated mouse albumin was tested by ELISA (A) with sera derived from non-immunized, OVA-, CaOVA- and AcOVA-immunized mice and shown with OD values. Results show representative data from two immunization experiments. Correlation between Ca-mouse albumin and Ac-mouse albumin was analyzed for both CaOVA- and AcOVA-immunized mice (B). Both immunization experiments are depicted (diamonds (n=5), circles (n=6 for CaOVA-immunized mice, n=5 for AcOVA-immunized mice)). Correlation coefficient is calculated with Spearman, $p < 0,05$ depicts significance. OVA, ovalbumin; Ca, carbamylated; Ac, acetylated; AU, arbritrary units; r, correlation coefficient; p, p-value

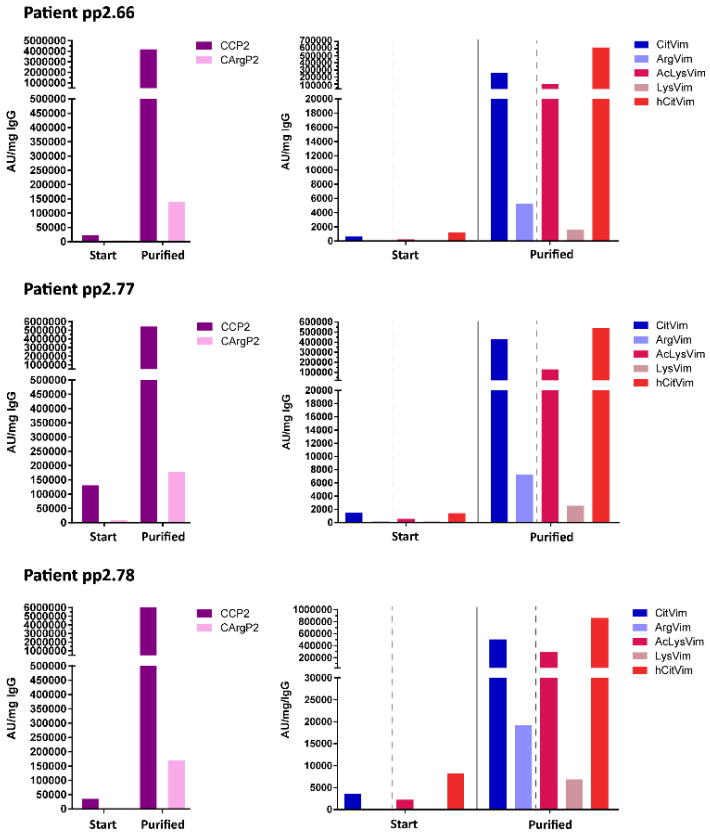
Cross-reactive antibodies towards different modifications are present in RA patients.

The data presented above were all obtained in mice and do not address whether also in humans, AMPA are cross-reactive towards different classes of modified antigens. Therefore, we next isolated ACPA-IgG from synovial fluid or plasma of 7 patients as previously described (17, 25). We focused on ACPA as the ACPA-response is the most prominent AMPA-response in RA. As depicted in figure 8 and B, ACPA-IgG were strongly enriched following isolation. Next, the purified ACPA-IgG were analyzed for their reactivity towards a citrullinated, carbamylated or acetylated peptide from vimentin. In all cases, purified ACPA also showed a highly enriched reactivity towards these differently modified peptides. These data indicate that ACPA-IgG from RA patients are not only cross-reactive towards carbamylated antigens as also observed previously (11), but that they can also recognize acetylated antigens. To analyze whether also anti-CarP antibodies display cross-reactivity towards different classes of PTMs, we next isolated anti-CarP antibodies from sera of 2 anti-CarP-positive patients. As shown in figure 6C, the isolated antibodies were highly enriched for anti-CarP-reactivity. Likewise, as observed for isolated ACPA, also purified anti-CarP antibodies showed strongly enriched reactivity towards different classes of modified antigen. Together, these data indicate that different families of human AMPA are cross-reactive towards different classes of modified antigens, including acetylated antigens.

A


Patient pp2.80
CCP2
CArgP2
AU/mg IgG
Start
Purified
CitVim
ArgVim
AcLysVim
LysVim
hCitVim
Patient pp2.98
Patient pp2.93
Patient pp2.86

B


Patient pp2.66
CCP2
CArgP2
AU/mg IgG
Start
Purified
CitVim
ArgVim
AcLysVim
LysVim
hCitVim
Patient pp2.77
Patient pp2.78
AU/mg/IgG

C

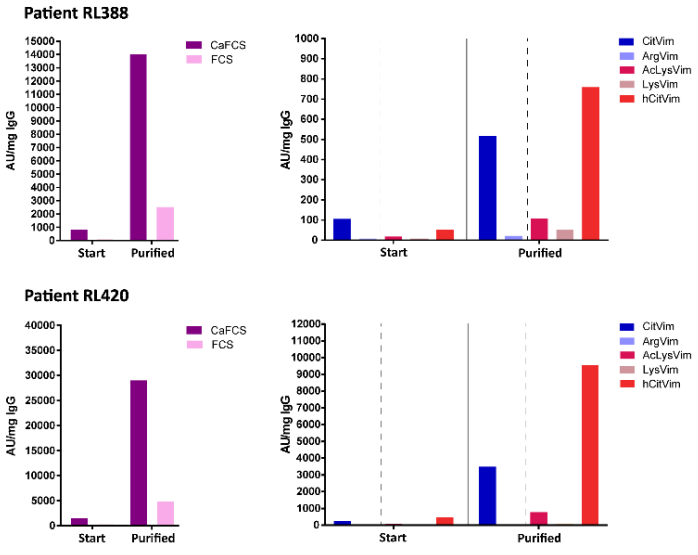

Patient RL388
CaFCS
FCS
AU/mg IgG
Start
Purified
CitVim
ArgVim
AcLysVim
LysVim
hCitVim
Patient RL420

Figure 8. Cross-reactivity of purified human ACPA or anti-CarP antibodies towards modified vimentin peptides. ACPA and anti-CarP antibodies were isolated from RA patients. ACPA from synovial fluid (A, n=4) and serum (B, n=3) from patients were tested on CCP2 and modified vimentin peptides. Anti-CarP antibodies from serum of RA patients (C, n=2) were tested on CaFCS and modified vimentin peptides. Reactivity is depicted as arbitrary units per mg IgG and calculated based on standards. CCP2, cyclic citrullinated peptide; CArgP2, cyclic arginine control peptide; Vim, vimentin peptide; Cit, citrullinated; Arg, arginine control; AcLys, acetylated lysine; Lys, lysine control; hCit, homocitrulline (carbamylated); FCS, fecal calf serum; Ca, carbamylated; AU/mg IgG, arbitrary units per milligram immunoglobulin G.

Discussion

Rheumatoid Arthritis is an autoimmune disease characterized by the presence of autoantibodies directed against different post-translationally modified antigens, including citrullinated, carbamylated and acetylated proteins. The origin of these different reactivities present in RA is still poorly understood. Here we show, by immunizing mice with a foreign protein carrying one defined PTM, that exposure to a protein carrying one particular PTM can lead to the induction of antibody-responses towards different PTMs. Likewise, we also show that AMPA from RA patients purified against one PTM can recognize different classes of PTMs. These findings are important as they indicate that the different AMPA-responses observed in RA patients can be derived from the same inciting antigen(s) carrying only one particular modification. Similarly, they provide a rationale for the presence of multiple AMPAs in RA, one of the hallmarks of disease.

Given the observations that different AMPAs target different antigens and are generally seen as distinct auto-antibody families, it has been intriguing to note that their presence often go together in RA. In contrast, AMPAs are less frequently present in other rheumatic diseases and their co-occurrence is rarely observed outside RA. The co-occurrence of different AMPAs represents an interesting conundrum as it is unclear why, after activation of a B-cell with a receptor for a particular modified protein, another B cell expressing a receptor recognizing a differently modified protein would also be activated in the same subject. In general, the activation of a particular B cell will not directly influence the activation of other B cells directed against other antigens, although it has been shown in a transgenic mouse model for SLE that epitope-spreading to other antigens can occur once tolerance is broken for one self-antigen (26).

Our data indicate that exposure to a defined antigen displaying a particular class of PTM, can lead to a cross-reactive antibody-response recognizing several classes of modified antigens, conceivably explaining the co-occurrence of multiple AMPA-reactivities in RA. In concordance with the data obtained in mice, our data indicate that also AMPA obtained from RA-patients are cross-reactive. It has been shown previously that anti-CarP-antibodies and ACPA can be cross-reactive towards respectively citrullinated and carbamylated antigens (11). Citrullination and carbamylation are highly similar, as they differ only one methyl-group, even though they are conversions from different amino acids. We now show that also acetylated antigens can be recognized by these antibodies. The latter observation was unexpected as, in contrast to homo-citrulline, acetylated-lysine shares less structural homology to citrulline as acetylation results in the substitution of the amine-group of lysine with a methyl-moiety. The cross-reactivity towards acetylated antigens was even more prominent in mice as the antibodies induced by CaOVA immunisation are unable to recognise citrullinated proteins, despite the similarity, even though they are able to recognise acetylated lysines. At present it is unclear how AMPA recognize antigens

harbouring these different PTMs at the molecular level, but it would be relevant to determine how the binding to these PTMs by an AMPA is accommodated by e.g. crystallization studies to obtain further molecular understanding of this interaction.

The finding that exposure to e.g. an acetylated protein leads to the formation of auto-antibodies against proteins carrying other classes of PTM as well, is also relevant for considerations on the breach of tolerance and induction of AMPA-responses. From our findings, it can be postulated that the inciting antigen responsible for the induction of e.g. ACPA or anti-CarP-antibodies does not have to be citrullinated or carbamylated, but could be represented by, for example, an acetylated protein. An increasing number of studies suggest that mucosal surfaces, specifically the periodontium, the gut and the lungs, as sites of disease initiation of RA and indicate the microbiome as an important driver of the initiation of auto-immunity. In this respect, especially protein–acetylation by bacteria might now also be incriminated in the induction of auto-antibody responses against PTM proteins. Recent evidence shows that many bacterial species are able to acetylate proteins (27), including bacteria proposed as link between periodontal infection and RA (28). Given our observation that AMPAs recognizing citrullinated and carbamylated proteins can be cross-reactive to acetylated proteins, these findings together provide a novel and stimulating angle to the notion that the microbiome contributes to the induction of auto-immunity in RA. Therefore, a logical next step is to test faecal extracts from RA patients for the presence of acetylated bacterial proteins to obtain more insight on the possible link between the microbiome, the presence of acetylated proteins, and RA.

Through the formation of acetylated proteins, disturbances of the microbiome (e.g. through infection) could lead to the formation of acetylated proteins detected by the immune system and thereby to the induction of AMPA-responses. In doing so, the origin of the T cell help required for the B cell to undergo isotype-switching and somatic hypermutation could come from different sources as, in this scenario, it is highly conceivable that microbe-specific T cells help the B cell initially recognizing the microbe-derived modified protein. Upon further somatic hypermutation, the B cell response could be selected/start recognizing other modified proteins explaining the cross-reactive nature of AMPAs and the observation that different AMPAs often appear together in patients. Likewise, the diversification towards other PTMs could, potentially, also explain the observation that the HLA-Shared-Epitope (SE) is associated with ACPA-positive RA, whereas the initial appearance of ACPA is HLA-SE- independent. Possibly, by diversification of an, initially, HLA-SE-independent AMPA-reaction, new HLA-SE-restricted T cells are recruited into this response, associated with ACPA-positive RA. Thus, in this scenario, the link to the microbiome, the cross-reactive nature of AMPAs, the breach of tolerance to self-modified proteins, including PTM modified histones as emerging autoantigens in RA, the HLA-Shared-Epitope-association with the “second hit”, as well as the concordant appearance of AMPAs in disease can be explained.

Our study has several limitations as we did not show that also in humans the inciting antigen carrying a particular PTM will lead to the induction of a cross-reactive AMPA-response. Obviously, studies immunizing a host with a defined modified antigen, as was performed in mice, is not feasible in humans and therefore the concepts obtained from such animal-studies will be difficult to demonstrate in the human system. Nonetheless, the observation that also human AMPAs are cross-reactive to several different PTM does support such views. Despite the advantages of using a controlled setting for the immunization of mice, a major pitfall of studying RA-associated antibodies in mice is the inability to induce the production of ACPAs with our standard immunization protocol, i.e. two subsequent immunisations in aluminium hydroxide. Consequently, the analysis of antibody cross-reactivity towards citrullinated antigens is limited and restricted to the human setting. In addition, our antibody experiments are focused on polyclonal antibody responses. Therefore, we are unable to predict the cross-reactivity on monoclonal level. Nevertheless, our inhibition studies do suggest that individual antibodies are capable of cross-recognising multiple PTM, though isolation of monoclonal antibodies will be necessary to validate this notion.

In conclusion, our data show that induction of antibodies in mice towards multiple PTM reactivities can be achieved by the encounter with a protein carrying one specific posttranslational modification. These studies indicate that the different AMPA present in RA could have a common “background” and provide novel insight into the origins of AMPAs, the most prominent biological hallmark of RA.

References

1. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010;62(9):2569-81.
2. Vossenaar ER, Zendman AJ, van Venrooij WJ, Pruijn GJ**.** PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. Bioessays. 2003;25(11):1106-18.
3. van der Woude D, Rantapaa-Dahlqvist S, Ioan-Facsinay A, Onnekink C, Schwarte CM, Verpoort KN, et al. Epitope spreading of the anti-citrullinated protein antibody response occurs before disease onset and is associated with the disease course of early arthritis. Ann Rheum Dis. 2010;69(8):1554-61.
4. van de Stadt LA, de Koning MH, van de Stadt RJ, Wolbink G, Dijkmans BA, Hamann D, et al. Development of the anti-citrullinated protein antibody repertoire prior to the onset of rheumatoid arthritis. Arthritis Rheum. 2011;63(11):3226-33.
5. Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. PLoS One. 2012;7(5):e35296.
6. Verpoort KN, Jol-van der Zijde CM, Papendrecht-van der Voort EA, Ioan-Facsinay A, Drijfhout JW, van Tol MJ, et al. Isotype distribution of anti-cyclic citrullinated peptide antibodies in undifferentiated arthritis and rheumatoid arthritis reflects an ongoing immune response. Arthritis Rheum. 2006;54(12):3799-808.
7. Titcombe PJ, Wigerblad G, Sippl N, Zhang N, Shmagel AK, Sahlstrom P, et al. Pathogenic Citrulline-Multispecific B Cell Receptor Clades in Rheumatoid Arthritis. Arthritis Rheumatol. 2018;70(12):1933-45.
8. Suwannalai P, van de Stadt LA, Radner H, Steiner G, El-Gabalawy HS, Zijde CM, et al. Avidity maturation of anti-citrullinated protein antibodies in rheumatoid arthritis. Arthritis Rheum. 2012;64(5):1323-8.
9. Trouw LA, Rispens T, Toes REM**.** Beyond citrullination: other post-translational protein modifications in rheumatoid arthritis. Nat Rev Rheumatol. 2017;13(6):331-9.
10. Shi J, van Veelen PA, Mahler M, Janssen GM, Drijfhout JW, Huizinga TW, et al. Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies. Autoimmun Rev. 2014;13(3):225-30.
11. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci U S A. 2011;108(42):17372-7.
12. Juarez M, Bang H, Hammar F, Reimer U, Dyke B, Sahbudin I, et al. Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis. Ann Rheum Dis. 2016;75(6):1099-107.
13. Guan KL, Yu W, Lin Y, Xiong Y, Zhao S**.** Generation of acetyllysine antibodies and affinity enrichment of acetylated peptides. Nat Protoc. 2010;5(9):1583-95.
14. Verheul MK, van Veelen PA, van Delft MAM, de Ru A, Janssen GMC, Rispens T, et al. Pitfalls in the detection of citrullination and carbamylation. Autoimmun Rev. 2018;17(2):136-41.

15. Suwannalai P, Scherer HU, van der Woude D, Ioan-Facsinay A, Jol-van der Zijde CM, van Tol MJ, et al. Anti-citrullinated protein antibodies have a low avidity compared with antibodies against recall antigens. Ann Rheum Dis. 2011;70(2):373-9.
16. Kerkman PF, Rombouts Y, van der Voort EI, Trouw LA, Huizinga TW, Toes RE, et al. Circulating plasmablasts/plasmacells as a source of anticitrullinated protein antibodies in patients with rheumatoid arthritis. Ann Rheum Dis. 2013;72(7):1259-63.
17. Rombouts Y, Willemze A, van Beers JJ, Shi J, Kerkman PF, van Toorn L, et al. Extensive glycosylation of ACPA-IgG variable domains modulates binding to citrullinated antigens in rheumatoid arthritis. Ann Rheum Dis. 2016;75(3):578-85.
18. Dekkers JS, Verheul MK, Stoop JN, Liu B, Ioan-Facsinay A, van Veelen PA, et al. Breach of autoreactive B cell tolerance by post-translationally modified proteins. Ann Rheum Dis. 2017;76(8):1449-57.
19. Stoop JN, Fischer A, Hayer S, Hegen M, Huizinga TW, Steiner G, et al. Anticarbamylated protein antibodies can be detected in animal models of arthritis that require active involvement of the adaptive immune system. Ann Rheum Dis. 2015;74(5):949-50.
20. Cantaert T, Teitsma C, Tak PP, Baeten D**.** Presence and role of anti-citrullinated protein antibodies in experimental arthritis models. Arthritis Rheum. 2013;65(4):939-48.
21. Hill JA, Bell DA, Brintnell W, Yue D, Wehrli B, Jevnikar AM, et al. Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. J Exp Med. 2008;205(4):967-79.
22. Kidd BA, Ho PP, Sharpe O, Zhao X, Tomooka BH, Kanter JL, et al. Epitope spreading to citrullinated antigens in mouse models of autoimmune arthritis and demyelination. Arthritis Res Ther. 2008;10(5):R119.
23. Pullen GR, Fitzgerald MG, Hosking CS**.** Antibody avidity determination by ELISA using thiocyanate elution. J Immunol Methods. 1986;86(1):83-7.
24. van Delft MAM, Verheul MK, Burgers LE, Rantapaa-Dahlqvist S, van der Helm-van Mil AHM, Huizinga TWJ, et al. The anti-carbamylated protein antibody response is of overall low avidity despite extensive isotype switching. Rheumatology (Oxford). 2018;57(9):1583-91.
25. Hafkenscheid L, Bondt A, Scherer HU, Huizinga TW, Wuhrer M, Toes RE, et al. Structural Analysis of Variable Domain Glycosylation of Anti-Citrullinated Protein Antibodies in Rheumatoid Arthritis Reveals the Presence of Highly Sialylated Glycans. Mol Cell Proteomics. 2017;16(2):278-87.
26. Degn SE, van der Poel CE, Firl DJ, Ayoglu B, Al Qureshah FA, Bajic G, et al. Clonal Evolution of Autoreactive Germinal Centers. Cell. 2017;170(5):913-26 e19.
27. Ouidir T, Kentache T, Hardouin J**.** Protein lysine acetylation in bacteria: Current state of the art. Proteomics. 2016;16(2):301-9.
28. Butler CA, Veith PD, Nieto MF, Dashper SG, Reynolds EC**.** Lysine acetylation is a common post-translational modification of key metabolic pathway enzymes of the anaerobe Porphyromonas gingivalis. J Proteomics. 2015;128:352-64.

PART II

AUTOIMMUNITY IN EARLY DISEASE

Hans Holbein der Jügere (1497-1543) Erasmus writing (detail), 1523, Le Louvre Paris, France

Chapter 6

Autoantibody Status is not Associated with Early Treatment Response to first-line Methotrexate in Patients with Early Rheumatoid Arthritis

Rheumatology (Oxford). 2019 Jan 1;58(1):149-153.

Jacqueline S. Dekkers[1], Sytske Anne Bergstra[1], Arvind Chopra[2], Mohammed Tikly[3], João Eurico Fonseca[4], Karen Salomon-Escoto[5], Tom W.J. Huizinga[1], Diane van der Woude[1]

1. Department Of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands
2. Centre For Rheumatic Diseases, Pune, India
3. Division Of Rheumatology, University of Witwatersrand, Johannesburg, South Africa.
4. Rheumatology Research Unit, Lisbon Academic Medical Centre, Portugal
5. Department Of Rheumatology, UMass Memorial Medical Center, Massachusetts, USA

ABSTRACT

Objectives

In rheumatoid arthritis (RA), the relationship between autoantibody status and treatment response to methotrexate remains unclear. We investigated the association between autoantibody status and early remission in newly diagnosed RA-patients treated with methotrexate using real-world data.

Methods

RA-patients initially treated with methotrexate were selected from an international observational database (METEOR). Patients were stratified into autoantibody-positive (rheumatoid factor (RF)- and/or anti-citrullinated-protein antibodies (ACPA)-positive) or autoantibody negative (RF- and ACPA- negative). The effect of autoantibody status on the chance of achieving remission within 3 to 6 months was analysed using Cox-proportional hazards regression.

Results

Data from 1826 RA-patients were available for analysis. DAS remission was achieved in 17% (318/1,826). This was similar in autoantibody-positive (17% (282/1629)) and -negative patients (18% (36/197)). Hence, autoantibody positivity was not associated with remission (HR0.89, 95%CI 0.57;1.38). Similar findings were found when stratified for methotrexate monotherapy (HR0.75, 95%CI 0.41;1.37) or combination treatment (HR0.76, 95%CI 0.37;1.54). Good physical function (HAQ<0.5) was achieved in 33% (530/1590) of all patients. Autoantibody-positivity was also not associated with HAQ<0.5 (HR1.05, 95%CI 0.71;1.57).

Conclusions

Autoantibody status is not associated with early remission in newly diagnosed RA-patients receiving methotrexate. This indicates that methotrexate is effective as initial treatment strategy regardless of autoantibody status.

Introduction

Rheumatoid arthritis (RA) is generally considered to consist of two separate entities: autoantibody-positive and -negative disease, each with distinct genetic and environmental risk factors and disease outcomes. Autoantibody-positive patients have worse long-term outcomes with less functional ability, worse disease activity and more radiographic joint damage(1-3). The presence of autoantibodies is associated with a better treatment response to rituximab(4) and abatacept(5), but whether an association exists with other (more commonly used) drugs, most notably methotrexate, remains unknown.

The presence of rheumatoid factor (RF) and/or anti-citrullinated protein antibodies (ACPA), especially at high levels, is mentioned as poor prognostic factor for treatment response to methotrexate in international treatment recommendations(6) even though the relationship between autoantibody status and treatment response to methotrexate is unclear. Some studies have suggested that autoantibody-positive patients might respond better to methotrexate, while other studies do not support this conclusion(7-10). In a previous cohort study disease-modifying antirheumatic drug (DMARD) treatment was equally effective in autoantibody-positive and -negative patients, but treatment differed and not all patients received methotrexate(11, 12). As methotrexate is the most widely used anti-rheumatic drug in clinical practice(6), it would be important to know whether the presence of autoantibodies is associated with better treatment response, since patients may benefit from treatment tailored to "autoantibody status". We therefore investigated the relationship between autoantibody status and remission in newly diagnosed RA-patients treated with first-line methotrexate.

Methods

Study population

Data were obtained from the METEOR register(13). This is an international, observational database of patients with a diagnosis of RA according to the rheumatologist, attending daily clinical practice. Data of RA-patients with symptom duration <5 years, baseline Disease Activity Score (DAS) >1.6 and follow-up visits after 3-6 months were selected. At least one of the following measures had to be available: DAS, DAS28, *Simplified Disease Activity Index* (*SDAI*), Clinical Disease Activity Index (CDAI), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) or health assessment questionnaire (HAQ). Data regarding autoantibody status (ACPA and/or RF, which were measured locally at the participating centers) had to be available and methotrexate had to be part of initial treatment. Data were gathered between 1995-2017 and contained irregular time intervals and different numbers of follow-up visits per patient due to the observational design of the database. All follow-up visits within 3 to 6 months after treatment initiation were selected.

Statistical analysis

Patients were stratified into autoantibody-positive (RF- and/or ACPA-positive) and -negative patients (RF- and ACPA-negative) and summary statistics were generated for baseline characteristics. Missing data were imputed using multivariate normal multiple imputation (30 imputations)(14). The variables patient-reported pain, ACPA and DAS contained most missing values (17 to 28% of values missing). Variables included for imputation were: year of birth, sex, RF, ACPA, CRP, ESR, weight, height, Ritchie articular index, SDAI, CDAI, erosions, HAQ, swollen joint count, DAS, DAS28, doctor's and patient's global assessment, and patient-reported pain. The effect of autoantibody status on the chance of achieving remission (DAS<1.6) or good physical function (HAQ<0.5) was analysed using Cox-proportional hazards regression with DAS or HAQ as time-dependent covariates(15) and correcting for confounders. Sub-analyses of methotrexate treatment strategies were performed. Patients were stratified into four groups according to initial medication strategy and we tested whether there was effect modification of these medication groups. In addition, we evaluated the presence of effect modification by testing for statistical interaction between autoantibody status and country. If no interactions were found (p>0.10), data of all countries were combined. Moreover, patients were stratified based symptom duration and assessed whether symptom duration was an effect modifier. Analyses were performed using StataSE14 (StataCorp LP).

Sensitivity analysis

Complete case analysis was performed as a sensitivity analysis. Linear mixed model (LMM) analyses were performed as sensitivity analyses to assess the effect of autoantibody status on DAS, HAQ and ESR as continuous outcomes during treatment with methotrexate. To account for irregular time intervals, random intercept and slope were added to each model. Interaction between time in follow-up and autoantibody status was added and adjusted for potential confounders, to assess whether the effect of autoantibody status on treatment outcome differed over time. Sensitivity analysis based on patients of European centers was performed to count for the dissimilarity between the frequency of seronegative patients between countries.

Results

Of the individuals registered in the METEOR database, 1,826 patients fulfilled the selection criteria. A flow-chart of patient selection is depicted in Figure 1. Patients originated from 20 different countries, with 93% of data originating from India, South-Africa, Portugal, the Netherlands, the United States, Mexico and Great Britain. 1,629 (89%) patients were autoantibody-positive (RF positive n=1,554, ACPA positive n=849) and 197 patients (11%) were autoantibody-negative. Baseline characteristics are depicted in Table 1. At baseline, autoantibody-positive patients were younger, had longer disease duration, higher baseline DAS and increased levels of acute-phase reactants ($p< 0.001$). Autoantibody-negative patients more often received methotrexate monotherapy and less frequently combination therapy with other conventional synthetic DMARDs compared to autoantibody-positive patients ($p< 0.001$).

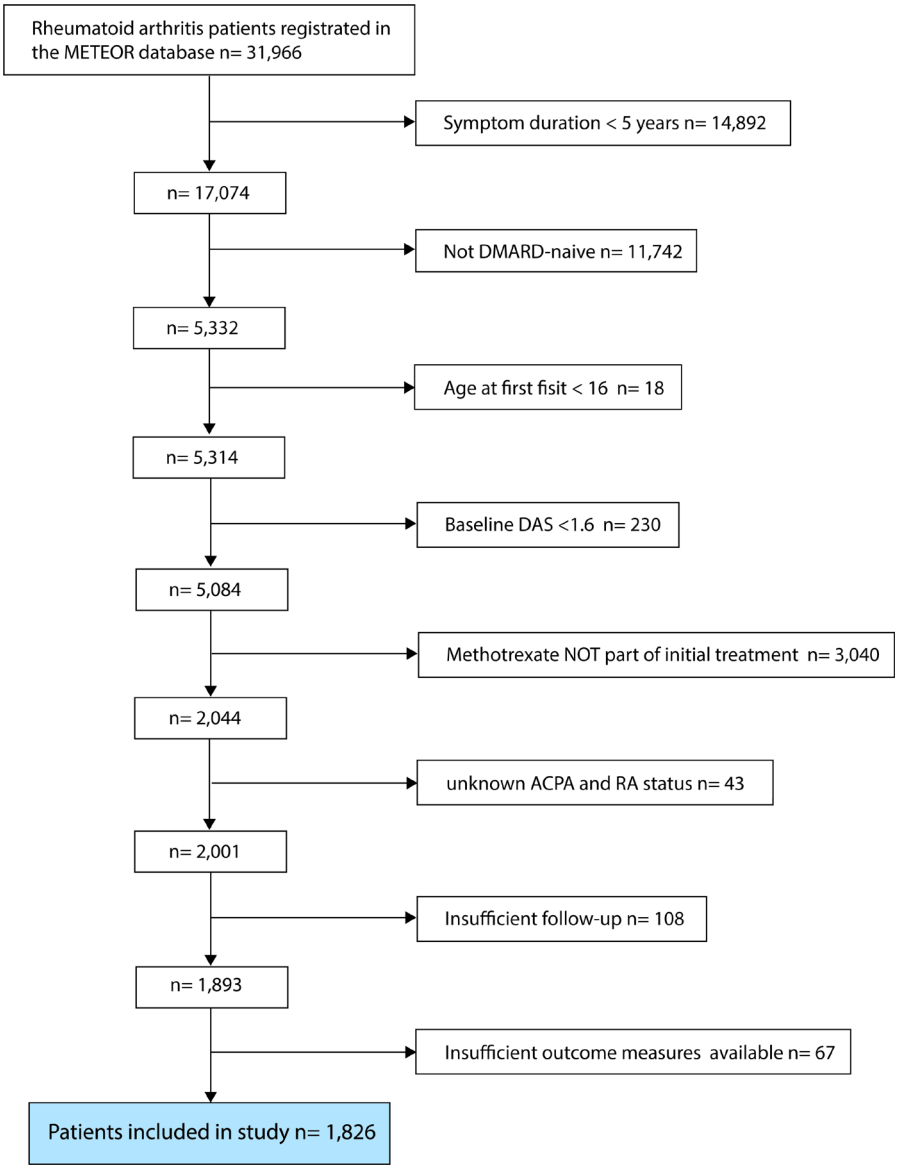


Figure 1. Flow-chart of patient selection. No effect modification of country on autoantibody status was present (p=0.62), therefore data of all countries were combined. In a period of 6 months, DAS remission was achieved by a similar percentage of autoantibody-positive (17%, 282/1629) and -negative (18%, 36/197) patients. The probability of achieving DAS remission over time is depicted for autoantibody-positive and negative RA-patients in Figure 2. Accounting for potential confounders, autoantibody positivity did not associate with remission (HR 0.89, 95%CI 0.57;1.38) (Table 2). Similar findings were found when stratified for methotrexate monotherapy (HR 0.75, 95%CI 0.41;1.37) or combination treatment (HR 0.76, 95%CI 0.37;1.54).

Table 1. Complete case analysis

DAS Remission	**Hazard ratio**	**95% CI for HR**		**p Value**
		Lower	**Upper**	
*A: Adjusted Model**	1.01	0.66	1.55	0.97
*B: Adjusted Model**	0.84	0.46	1.54	0.57
*C: Adjusted Model**	0.78	0.40	1.53	0.47
HAQ <0.5	**Hazard ratio**	**95% CI for HR**		**p Value**
		Lower	**Upper**	
E: Adjusted Model*	1.13	0.78	1.63	0.51
F: Adjusted Model*	0.95	0.54	1.67	0.86
G: Adjusted Model*	1.38	0.82	2.34	0.22

Complete case analysis based on cox-proportional hazard regression analysis. Selection of covariates for adjustment was based on potential confouding variables with uneven distribution and change-in-estimation (e.g. significant change in OR). Potential counfouding variables tested: sex, age, symptom duration at diagnosis, follow-up duration, DAS at baseline, HAQ at baseline, country, smoking and medication.

A/E: association between autoantibody positivity and DAS remission/HAQ<0.5

B/F: methotrexate monotherapy stratum

C/G: combinationtherapy stratum

*Adjusted for sex, symptom duration at diagnosis, country, smoking, methotrexate treatment strategies and DAS/HAQ at baseline

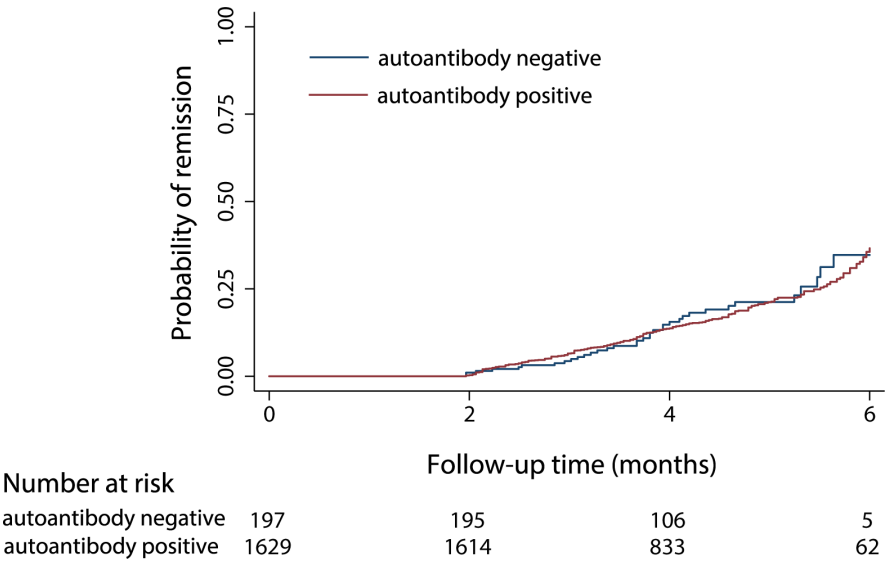


Figure 2. Probability of achieving remission over time. Newly diagnosed RA-patients were analysed for probability of achieving remission (DAS < 1.6). Month 0 corresponds to the time of starting initial treatment with methotrexate mono- or combination therapy. Autoantibody-positive patients (ACPA and/or RF) (red line, n=1,629) and autoantibody-negative patients (ACPA and RF negative) (blue line, n=197) are depicted. The depicted figure is based on non-imputed data. RA, rheumatoid arthritis; DAS, disease activity score; ACPA, anti-citrullinated protein antibodies; RF, rheumatoid factor.

Regarding good physical function (HAQ < 0.5), 236 patients had good physical function at baseline and an additional 33% (530/1590) achieved this outcome within 6 months. Percentages were comparable for autoantibody-positive (35%, 491/1413) and -negative (33%, 39/117) patients. Thus, autoantibody positivity did not associate with HAQ<0.5 (HR1.05, 95%CI 0.71;1.57). Complete case sensitivity analysis showed very similar results as the analysis based on imputed data (Table 3). Additional analyses based on LMM revealed no differences between autoantibody-positive and -negative patients regarding DAS (p=0.71), HAQ (p=0.59) or ESR (p=0.27). Sub-analyses of methotrexate treatment strategies revealed no presence of effect modification by medication strategy (p>0.10). In addition, we stratified based on symptom duration and assessed whether symptom duration was an effect modifier, but also did not find such an effect (p=0.22).

Although ACPA and RF often co-occur, RF testing has been used longer and is in some countries still determined more often than ACPA. We therefore performed a sensitivity analysis to evaluate the association between RF-positivity and short-term remission. In line with our previous findings, RF-positivity alone did not associate with short-term DAS remission nor the ability to regain function (Table 4). Additional sensitivity analysis based on patients of European centers only, revealed similar results (Table 5).

Table 2. Patient characteristics at baseline

	All RA-patients n=1,826		***Autoantibody-positive n=1,629***		***Autoantibody-negative n=197***		
Characteristic	Values available	Summary statistics	Values available	Summary statistics	Values available	Summary statistics	p-value
ACPA (positivity), n (%)	1,121	849 (75)	924	849 (92)	197	0 (0)	<0.001
RF (positivity), n (%)	1,810	1,554 (85)	1,613	1,554 (96)	197	0 (0)	<0.001
Gender (female), n (%)	1,814	1,429 (79)	1,620	1,288 (80)	194	141 (73)	0.03
Age at diagnosis (years), mean ±SD	1,815	48±13	1,619	47±13	197	54±16	<0.001
Symptom duration (months), median (IQR)	1,826	15 (6;36)	1,629	18 (7;36)	197	7 (3;17)	<0.001
Visit count, mean ±SD	4,265	3.14±1.05	3,782	3.13±1.02	483	3.27±1.24	0.13
Follow-up duration (months), mean ±SD	1,826	4.2±1.2	1,629	4.2±1.2	197	4.2±1.1	0.98
Cigarette smoking, n (%)	1,602		1,461		141		<0.001
Never		1,353 (85)		1,250 (86)		103 (73)	
Current		158 (10)		140 (10)		18 (13)	
Past		91 (6)		71 (5)		20 (14)	
ESR (mm/hr), median (IQR)	1,588	51 (29;85)	1,413	55 (31;85)	175	30 (15;48)	<0.001
CRP (mg/dl), median (IQR)	1,498	23 (9;49)	1,324	24 (11;52)	154	10 (3;24)	<0.001
VAS, median (IQR)	1,357	50 (50;75)	1,212	50 (50;75)	145	50 (35;75)	0.59
SJC in 44 joints, median (IQR)	1,664	5 (2;10)	1,492	5 (2;10)	172	6 (3;11)	0.12
RAI, median (IQR)	1,661	9 (5;16)	1,489	10 (5;16)	172	6 (4;9.5)	0.13
DAS, mean ±SD	1,078	3.8±1.1	979	3.9±1.0	117	3.4±1.1	0.35
HAQ, median (IQR)	1,505	1.0 (0.6;1.6)	1,384	1.0 (0.6;1.6)	121	1.1 (0.5;1.6)	0.32
First-line treatment strategy:							
MTX monotherapy, n (%)	1,826	653 (36)	1,629	549 (34)	197	104 (53)	
MTX & csDMARD & glucocorticoid , n (%)	1,826	806 (44)	1,629	728 (45)	197	78 (40)	
MTX & synthetic DMARD, n (%)	1,826	351 (19)	1,629	338 (21)	197	13 (7)	
MTX & biological DMARD, n (%)	1,826	16 (<1)	1,629	14 (<1)	197	2 (<1)	

All data are mean and SD or median and interquartile range (IRQ, 25th ; 75th percentile). ACPA, anti-citrullinated protein antibody; RF, rheumatoid factor, ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; VAS, visual analogue scale general health patient; SJC, swollen joint count on a 44-joint count; RAI, Ritchie articular index on a 53 joint count; DAS, disease activity scores (ESR) on a 44-joint count; HAQ, Health Assessment Questionnaire; SDAI, Simplified Disease Activity Index; MTX, methotrexate; DMARD, disease modifying anti-rheumatic drugs.

Table 3. Cox-proportional hazard regression analysis

		95% CI for HR		
DAS Remission	**Hazard ratio**	**Lower**	**Upper**	**p Value**
*A: Adjusted Model**	0.89	0.57	1.38	0.61
B: Crude Model	0.96	0.67	1.35	0.79
*C: Adjusted Model***	0.75	0.41	1.37	0.35
D: Crude Model	0.88	0.56	1.38	0.57
*E: Adjusted Model***	0.76	0.37	1.54	0.45
F: Crude Model	1.26	0.72	2.21	0.42

		95% CI for HR		
HAQ <0.5	**Hazard ratio**	**Lower**	**Upper**	**p Value**
*A: Adjusted Model**	1.05	0.71	1.57	0.80
B: Crude Model	1.65	1.19	2.28	<0.01
*C: Adjusted Model***	0.79	0.44	1.42	0.43
D: Crude Model	2.02	1.26	3.25	<0.01
*E: Adjusted Model***	1.28	0.74	2.22	0.38
F: Crude Model	1.65	1.03	2.66	0.04

Selection of covariates for adjustment was based on potential confouding variables with uneven distribution and change-in-estimation (e.g. significant change in OR). Potential confouding variables tested: sex, age, symptom duration at diagnosis, follow-up duration, DAS at baseline, HAQ at baseline, country, smoking and medication.

A/B: association between autoantibody positivity and DAS remission/HAQ<0.5.

C/D: methotrexate monotherapy stratum.

E/F: combination therapy stratum

*Adjusted for sex, symptom duration at diagnosis, country, smoking, methotrexate treatment strategies and DAS/HAQ at baseline

**Adjusted for sex, symptom duration at diagnosis, country, smoking and DAS/HAQ at baseline

Table 4. Cox-proportional hazard regression analysis for rheumatoid factor

		95% CI for HR		
DAS Remission	**Hazard ratio**	**Lower**	**Upper**	**p Value**
*A: Adjusted Model**	1.05	0.70	1.58	0.81
B: Crude Model	0.96	0.70	1.32	0.82
*C: Adjusted Model***	1.05	0.55	2.00	0.89
D: Crude Model	0.83	0.55	1.25	0.37
*E: Adjusted Model***	0.95	0.50	1.79	0.87
F: Crude Model	1.31	0.79	2.15	0.29

		95% CI for HR		
HAQ <0.5	**Hazard ratio**	**Lower**	**Upper**	**p Value**
*C: Adjusted Model**	1.14	0.82	1.60	0.44
D: Crude Model	1.39	1.06	1.83	0.02
*C: Adjusted Model***	0.86	0.50	1.48	0.59
D: Crude Model	1.90	1.25	2.90	<0.01
*E: Adjusted Model***	1.31	0.85	2.02	0.22
F: Crude Model	1.26	0.87	1.82	0.23

Selection of covariates for adjustment was based on potential confouding variables with uneven distribution and change-in-estimation (e.g. significant change in OR). Potential counfouding variables tested: sex, age, symptom duration at diagnosis, follow-up duration, DAS at baseline, HAQ at baseline, country, smoking and medication.

A/B: association between rheumatoid factor positivity and DAS remission/HAQ<0.5.

C/D: methotrexate monotherapy stratum.

E/F: combinationt herapy stratum

*Adjusted for sex, symptom duration at diagnosis, country, smoking, methotrexate treatment strategies and DAS/HAQ at baseline

**Adjusted for sex, symptom duration at diagnosis, country, smoking and DAS/HAQ at baseline

Table 5. Cox-proportional hazard regression analysis: sensitivity analysis based on European countries

		95% CI for HR		
DAS Remission	**Hazard ratio**	**Lower**	**Upper**	**p Value**
A: Adjusted Model*	1.09	0.53	2.25	0.81

		95% CI for HR		
HAQ <0.5	**Hazard ratio**	**Lower**	**Upper**	**p Value**
B: Adjusted Model*	1.44	0.63	3.26	0.38

A/B: association between autoantibody positivity and DAS remission/HAQ<0.5

*Adjusted for sex, symptom duration at diagnosis, country, smoking, methotrexate treatment strategies and DAS/HAQ at baseline

Discussion

This study reveals that autoantibody status does not associate with short-term DAS remission and good physical function in newly diagnosed RA-patients receiving methotrexate in a real-world setting. Autoantibody-positive RA-patients present with more severe disease (higher baseline disease activity and increased levels of acute-phase reactants) compared to autoantibody-negative patients. We found that 17% of newly diagnosed RA-patients achieved DAS remission, independent of autoantibody status. In accordance, the percentage of patients achieving a good physical function was independent of the autoantibody status. Together, these findings indicate that methotrexate is effective as initial treatment strategy regardless of autoantibody status.

Previous intervention studies suggested that the presence of ACPA in early RA-patients may be associated with a better response to methotrexate, with higher levels indicating an improved response (10, 16, 17). Moreover, ACPA-positive undifferentiated arthritis patients receiving methotrexate were found to be less likely to progress to RA[10]. A possible explanation for the discrepancies between these previous findings and our data may be related to differences in study populations, with the previous randomized controlled trials including only a selection of RA patients with high baseline disease activity. A strength of our study is that it is based on a large international real-world cohort that best mimics routine clinical practice. Our findings are in line with the results of a previous cohort study reporting equal treatment responses independent of autoantibody status in patients receiving various different initial DMARDs (11).

This study has several limitations. As the METEOR database is an observational database gathered during daily clinical practice most variables contain missing values. Data regarding ACPA status were more often missing compared to RF status, which may be explained by the time period of inclusion. To account for missing data we applied multiple imputations (14). Reciprocal analysis based on non-imputed data revealed no differences between autoantibody-positive and -negative patients. Another concern with real-world data is the variation in clinical scoring and a higher noise to signal ratio. It is to be expected that differences in DAS or other outcome measurements exist between different centres. However, it is less likely that measurements within one centre differ between autoantibody-positive and -negative individuals. Due to the observational nature of the database, we had to take several precautions to limit the influence of potential bias - multiple imputation, testing for effect modification and adjusting for potential confounders – but it is always possible that residual confounding remains.

A striking feature of our dataset was the high percentage of autoantibody-positive RA-patients (up to 89%), which is higher than in European cohorts but consistent with other international cohorts (11, 18). Some countries, particularly those with limited financial resources, included only a very limited number of autoantibody-negative patients, perhaps

because these patients are less frequently referred to rheumatologists in those settings. Never smokers were more prevalent among autoantibody positive RA patients. This is remarkable, since smoking is associated with ACPA-positive RA(19).This can be explained by the large proportion of Indian patients included in the analyses. The majority of these patients were autoantibody positive. After exclusion of Indian patients, 54% (350/651) of autoantibody positive patients were never smokers compared to 86% (1,250/1,461) found in the whole dataset. It has been previously published that especially the number of women smoking in India is low (approximately 3%)(20). Furthermore, the use of smokeless tobacco products is relatively high in India, which was not captured in this database (21).

In our dataset, information regarding autoantibody status was limited to ACPA and RF serology. We cannot exclude that some of the autoantibody-negative patients express other RA-associated serological markers such as anti-carbamylated protein antibodies or anti-acetylated protein antibodies (22). However, these novel autoantibodies are expected to be present in only a limited proportion of the ACPA- and RF-negative population.

In conclusion, we found that autoantibody status was not associated with early remission in newly diagnosed RA-patients receiving methotrexate in real-world clinical practice. The results from our study therefore do not support the hypothesis that treatment should be tailored to “autoantibody status” when it comes to initiating methotrexate therapy as first-line anti-rheumatic treatment. Rather, our results indicate that that methotrexate is effective as initial treatment strategy regardless of autoantibody status.

References

1. van der Helm-van Mil AH, Verpoort KN, Breedveld FC, Toes RE, Huizinga TW**.** Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. Arthritis Res Ther. 2005;7(5):R949-58.
2. Forslind K, Ahlmen M, Eberhardt K, Hafstrom I, Svensson B**.** Prediction of radiological outcome in early rheumatoid arthritis in clinical practice: role of antibodies to citrullinated peptides (anti-CCP). Ann Rheum Dis. 2004;63(9):1090-5.
3. Ronnelid J, Wick MC, Lampa J, Lindblad S, Nordmark B, Klareskog L, et al. Longitudinal analysis of citrullinated protein/peptide antibodies (anti-CP) during 5 year follow up in early rheumatoid arthritis: anti-CP status predicts worse disease activity and greater radiological progression. Ann Rheum Dis. 2005;64(12):1744-9.
4. Isaacs JD, Cohen SB, Emery P, Tak PP, Wang J, Lei G, et al. Effect of baseline rheumatoid factor and anticitrullinated peptide antibody serotype on rituximab clinical response: a meta-analysis. Ann Rheum Dis. 2013;72(3):329-36.
5. Gottenberg JE, Courvoisier DS, Hernandez MV, Iannone F, Lie E, Canhao H, et al. Brief Report: Association of Rheumatoid Factor and Anti-Citrullinated Protein Antibody Positivity With Better Effectiveness of Abatacept: Results From the Pan-European Registry Analysis. Arthritis Rheumatol. 2016;68(6):1346-52.
6. Smolen JS, Landewe R, Breedveld FC, Dougados M, Emery P, Gaujoux-Viala C, et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs. Ann Rheum Dis. 2010;69(6):964-75.
7. Katchamart W, Johnson S, Lin HJ, Phumethum V, Salliot C, Bombardier C**.** Predictors for remission in rheumatoid arthritis patients: A systematic review. Arthritis Care Res (Hoboken). 2010;62(8):1128-43.
8. Pease CT, Bhakta BB, Devlin J, Emery P**.** Does the age of onset of rheumatoid arthritis influence phenotype?: a prospective study of outcome and prognostic factors. Rheumatology (Oxford). 1999;38(3):228-34.
9. Verstappen SM, van Albada-Kuipers GA, Bijlsma JW, Blaauw AA, Schenk Y, Haanen HC, et al. A good response to early DMARD treatment of patients with rheumatoid arthritis in the first year predicts remission during follow up. Ann Rheum Dis. 2005;64(1):38-43.
10. van Dongen H, van Aken J, Lard LR, Visser K, Ronday HK, Hulsmans HM, et al. Efficacy of methotrexate treatment in patients with probable rheumatoid arthritis: a double-blind, randomized, placebo-controlled trial. Arthritis Rheum. 2007;56(5):1424-32.
11. Barra L, Pope JE, Orav JE, Boire G, Haraoui B, Hitchon C, et al. Prognosis of seronegative patients in a large prospective cohort of patients with early inflammatory arthritis. J Rheumatol. 2014;41(12):2361-9.
12. Kuriya B, Xiong J, Boire G, Haraoui B, Hitchon C, Pope J, et al. Earlier time to remission predicts sustained clinical remission in early rheumatoid arthritis--results from the Canadian Early Arthritis Cohort (CATCH). J Rheumatol. 2014;41(11):2161-6.
13. van den Berg R, van der Heijde D, Landewe R, van LK, Huizinga T**.** The METEOR initiative: the way forward for optimal, worldwide data integration to improve care for RA patients. Clin.Exp.Rheumatol. 2014;32(5 Suppl 85):S-40.

14. Horton NJ, Lipsitz SR**.** Multiple Imputation in Practice. The American Statistician. 2001;55(3):244-54.
15. Wells GA, Boers M, Shea B, Brooks PM, Simon LS, Strand CV, et al. Minimal disease activity for rheumatoid arthritis: a preliminary definition. J Rheumatol. 2005;32(10):2016-24.
16. Visser K, Verpoort KN, van Dongen H, van der Kooij SM, Allaart CF, Toes RE, et al. Pretreatment serum levels of anti-cyclic citrullinated peptide antibodies are associated with the response to methotrexate in recent-onset arthritis. Ann Rheum Dis. 2008;67(8):1194-5.
17. Wevers-de Boer K, Visser K, Heimans L, Ronday HK, Molenaar E, Groenendael JH, et al. Remission induction therapy with methotrexate and prednisone in patients with early rheumatoid and undifferentiated arthritis (the IMPROVED study). Ann Rheum Dis. 2012;71(9):1472-7.
18. Peschken CA, Hitchon CA, Robinson DB, Smolik I, Barnabe CR, et al. Rheumatoid arthritis in a north american native population: longitudinal followup and comparison with a white population. J Rheumatol. 2010;37(8):1589-95.
19. Kallberg H, Ding B, Padyukov L, , Ronnelid J, Klareskog L, et al. Smoking is a major preventable risk factor for rheumatoid arthritis: estimations of risks after various exposures to cigarette smoke. Ann Rheum Dis. 2011;70(3):508-11.
20. World Lung Foundation, tobacco atlas 2015.
21. Mishra GA, Pimple SA, Shastri SS**.** An overview of the tobacco problem in India. Indian J Med Paediatr Oncol. 2012;33(3):139-45.
22. Trouw LA, Rispens T, Toes REM**.** Beyond citrullination: other post-translational protein modifications in rheumatoid arthritis. Nat Rev Rheumatol. 2017;13(6):331-9.

Chapter 7

Possibilities for preventive treatment in Rheumatoid Arthritis? Lessons from Experimental Animal Models of Arthritis: a Systematic Literature Review and Meta-analysis

Annals of the Rheumatic Diseases. 2017 Feb;76(2):458-467.

Dekkers J.S.[1], Schoones J.W. [2], Huizinga T.W.[1], Toes R.E.[1], van der Helm-van Mil A.H. [1]

1. Department of Rheumatology Leiden University Medical Center, Leiden, The Netherlands
2. Walaeus Library, Leiden University Medical Center, Leiden, The Netherlands

ABSTRACT

Objective

Current research in rheumatoid arthritis focusses on preclinical disease phases as it is hypothesized that early preclinical treatment might prevent progression to full-blown disease. Since performance of studies in pre-arthritis phases in humans is challenging, animal models offer an opportunity to evaluate preventive treatments. We performed a systematic literature review and summarized treatment effects during different stages of arthritis development in animal models.

Methods

Eight medical literature databases were systematically searched. Studies were selected if they reported effects of synthetic or biological disease-modifying anti-rheumatic drugs in animal models of arthritis (collagen-induced arthritis and adjuvant-induced arthritis) on arthritis severity, as measured with arthritis severity scores, paw swelling or paw volume. Quality was assessed using an eleven item checklist. Study characteristics were extracted and effect sizes obtained in high-quality studies were summarized in meta-analyses. Studies were categorized in three groups; prophylactic (prior to generation of autoantibody response), pre-arthritis (after induction of autoantibody response) and therapeutic intervention (after arthritis development).

Results

Out of 1415 screened articles, 22 studies (including n=712 animals) were eligible, of good quality and included in meta-analyses. Prophylactic (16 experiments, n=312 animals) and pre-arthritis treatment (9 experiments, n=156 animals) both were associated with a reduction of arthritis severity ($p<0.001$ and $p=0.005$ respectively). Stratified analyses for different anti-rheumatic drugs initiated in the pre-arthritis phase suggested higher efficacy of methotrexate than of anti-TNF.

Conclusions

Data of experimental studies in animal models of arthritis suggest that prophylactic and pre-arthritis treatment strategies are effective and hint at differences in efficacy between anti-rheumatic drugs.

Introduction

During recent years, research in the field of rheumatoid arthritis (RA) has focused on the earliest stages of the disease. This has provided novel insights into the immunological processes that precede the transition from healthy to established disease. Currently, the field of RA is moving from disease suppressive treatments to prevention strategies, focussing on initiation of treatment in pre-arthritis phases. Within RA it has been shown that a prolonged symptom duration at treatment start is associated with a worse disease outcome, including a lower chance on achieving disease modifying anti-rheumatic drug (DMARD)-free sustained remission [1-4]. Therefore it is hypothesized that treatment initiation during pre-arthritis stages might result in an improved efficacy in preventing disease chronicity [5]. Within type I diabetes preventive trials have shown efficacy of very early intervention to prevent full blown disease [6-8].

To evaluate of very early treatment initiation can also prevent the development of RA, several placebo-controlled randomized controlled clinical trials have been initiated recently. These clinical trial studies will investigate the therapeutic potential of several immunomodulatory agents such as rituximab (PRAIRI study: NTR No. 1969), abatacept (APIPPRA study: ISRCTN No. 46017566 and ARIAA study: EudraCT No. 2014-000555-93), hydroxychloroquine (StopRA trial; NCT No. 02603146) and methotrexate (TREAT EARLIER; NTR No. 4853) in individuals at a risk of RA. A study evaluating the immunomodulatory effect of atorvastatin in seropositive arthralgia patients has also been initiated (STAPRA study: NTR No. 22389). Interestingly, design of these trials does not follow the normal development of phase 1,2 and subsequently phase 3 trials. Moreover, dosages used are from other indications targeting different biology and the length of treatment is relatively randomly defined. The performance of randomized clinical trials on preventative treatment strategies in individuals at risk for RA is therefore difficult to interpret. Early identification and recruitment of patients at risk for RA is difficult and execution of these studies is time consuming. Altogether, it will take several years before the majority of these currently ongoing clinical trials are completed and the results are known. Likewise, because of difference in study-design and patient selection it will be difficult to compare side-by-side which intervention will be most effective in reaching sustained symptom- and drug-free benefit.

Animal models of arthritis can function as innovative tools to study the potential of preventive therapies. Animal studies provide an opportunity to study the developing (auto)immune response at a very early disease phase and the translation of different stages of experimental arthritis development to the evolution of human disease might provide valuable information regarding possibilities of disease prevention. Murine models of arthritis are scientifically well defined, highly reproducible, genetic identical, represent different disease pathways relevant for RA and are readily available. While current animal

research mainly focusses on testing anti-rheumatic drugs in established disease, it is unknown whether preventive treatment in mouse models is also effective. In addition, it is unknown if the efficacy of prophylactic or pre-arthritis intervention depends on the type of anti-rheumatic drug used. These questions prompted us to perform a systematic literature review and summarize the knowledge on the efficacy of treatment initiated before arthritis was clinically evident. Our first aim was to evaluate if prophylactic or pre-arthritis treatment is effective in animal models of arthritis. The second aim was to evaluate the efficacy of different synthetic and biological DMARDs treatments initiated in pre-arthritis phases.

We focused on two widely accepted experimental models for RA; collagen-induced arthritis (CIA) and adjuvant-induced arthritis (AIA), both models are based on immunization-induced arthritis. In the CIA model, arthritis is induced by immunizations with cartilage proteins causing a break of tolerance and an immune-mediated inflammatory attack on the joints [9 10]. Animals receive type II collagen emulsified in complete Freund's adjuvant typically followed by a second injection three weeks later, leading to the development of chronic destructive arthritis. There are three developmental stages of arthritis in the CIA model; an induction phase, a pre-arthritis phase were auto-immunity is present in the absence of clinical symptoms, and established arthritis [11]. The AIA-model requires intradermal immunization with mycobacterial cell wall components suspended in mineral oil causing an acute and systemic inflammation. The development of arthritis in the AIA-model is thought to depend on a heat shock protein specific T-cell response. In contrast to CIA, AIA is self-limiting and contains three developmental stages of arthritis; incubation phase, pre-arthritis phase and a peak phase followed by a gradual regression of inflammation [12]. Thus, these experimental models of arthritis have clearly defined developmental stages that can be identified by immunological disease markers. According to these developmental stages of arthritis we discerned three types of treatment; prophylactic (prior to generation of autoantibodies), pre-arthritis (after induction of an autoantibody response) and therapeutic intervention (after arthritis development) (Figure 1). We performed a systematic literature review and summarized the effects of treatment initiated at these stages.

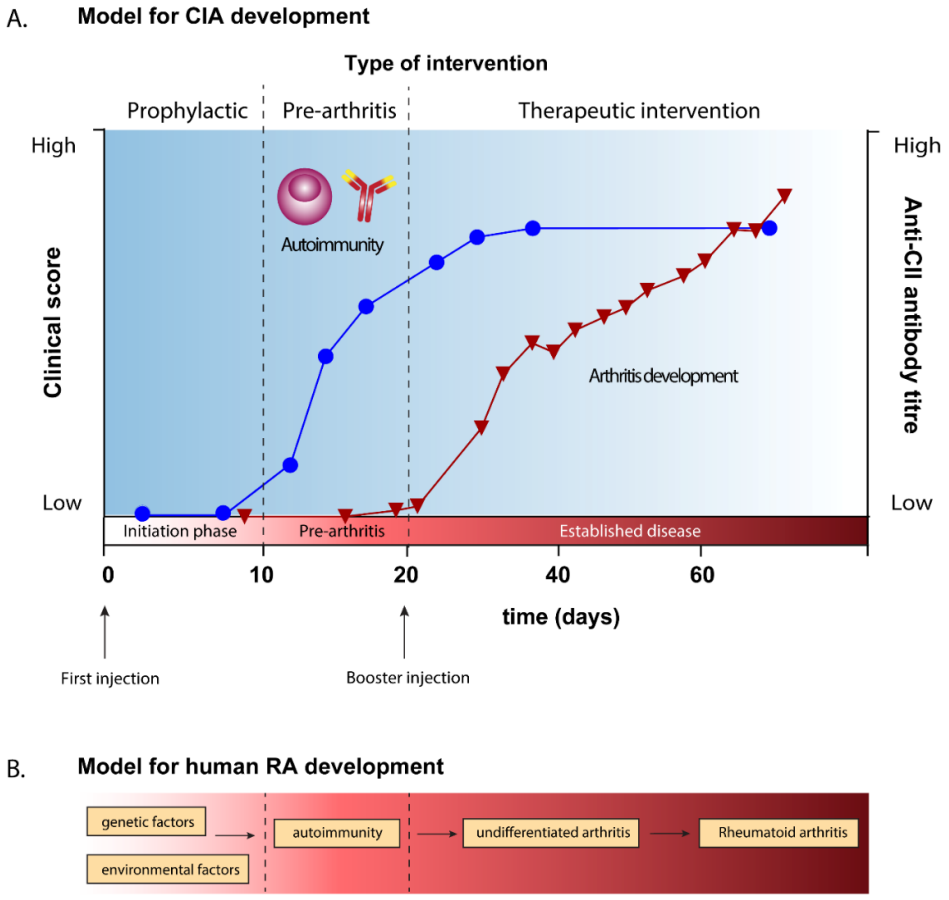


Figure 1. Schematic representation of developmental stages of collagen-induced arthritis (CIA) and human RA. Type of intervention during different developmental stages of experimental arthritis in the collagen-induced arthritis (CIA) model. Arthritis is generally induced by immunization with CII emulsified in complete Freund's adjuvant followed a booster injection three weeks later of CII in incomplete Freund's adjuvant. After immunization with type II collagen, during the initiation phase, auto-immunity towards collagen will develop (blue circles). Drug intervention during this time period is referred to as prophylactic treatment. This first stage is followed by a pre-arthritis phase which is characterized by the onset of autoimmunity and is marked by the development of autoantibodies against type II collagen which occurs around day 10. The time period in which auto-immunity is present and arthritis is still absent is referred to as the pre-arthritis period. Intervention during this pre-arthritis disease stage is described as pre-arthritis treatment. The time period of pre-arthritis arthritis is followed by the onset of arthritis (red triangles), which occurs around day 20 and leads to chronic destructive arthritis. Drug intervention at the established arthritis stage is described as therapeutic intervention (A). Schematic representation of human RA development. Genetic and environmental factors drive the onset of auto-immunity which subsequently leads to undifferentiated arthritis and finally full-blown disease (B).

Methods

Search strategy

Eight bibliographic databases (PubMed, Medline, Embase, Web of Science, Cochrane Library, CINAHL, Academic Search Premier and Science Direct), were searched to identify studies investigating treatment strategies in animal models for experimental arthritis (search conducted April 14, 2016). A systematic search strategy was developed for PubMed (see online supplementary file 1) and was subsequently applied in all other databases. Search terms were: rheumatoid arthritis, experimental models for arthritis, therapeutic intervention DMARDs (Methotrexate, Leflunomide, Cyclosporine, Sulfasalazine, Azathioprine or Hydroxychloroquine, Prednisolone) or biologicals (anti-TNF, Anti-IL-1, CTLA4-Ig, anti-IL-6 or anti-CD20) combined with the Boolean operators AND/OR.

Inclusion and exclusion criteria

The inclusion criteria are described in table 1. In short, we included published peer-reviewed studies reporting the effect of therapeutic intervention (synthetic or biological DMARDs) in the most commonly used in vivo models of experimental arthritis (CIA and AIA). Furthermore experiments should be prospectively controlled and information on clinical outcome and joint structural changes should be provided. Abstracts were assessed based on type of research (animal studies), drugs tested, outcome measures (arthritis severity) and duplicates. Full papers were assessed to identify experimental arthritis models, study design, treatment and control groups, therapeutic interventions and outcomes measurements used in the studies.

Table 1. Inclusion criteria for experimental studies reporting:

1. prospective controlled experiments using small animals
2. treatment group with experimentally induced arthritis CIA or AIA model
3. matched control group of animals with induced arthritis which receive control treatment (placebo) or animals with arthritis without any intervention.
4. testing of anti-rheumatic drugs; synthetic DMARDs (*Methotrexate, Leflunomide, Cyclosporine, Sulfasalazine, Azathioprine, Hydroxychloroquine or Prednisolone/Dexamethasone*) or biological DMARDs (*anti-TNF, anti-IL-1, CTLA4-Ig, anti-IL-6 or anti-CD20 monoclonal antibodies)*
5. effects on clinical outcome defined as arthritis severity score, paw swelling or paw volume
6. effects on joint structural changes: histological- (synovial hyperplasia, cell infiltration, pannus formation, oedema, fibrosis, cartilage and bone destruction) or radiological scores (X-ray or microCT)

Outcome measurements

Furthermore, studies were only selected if the following clinical outcomes were evaluated: arthritis severity scores (ordinal scale), paw swelling (mm) or paw volume (ml), or outcome measurements for joint structural changes by quantitative histological or radiographic scores measured with X-ray or microCT. An overview of the outcome measurements is presented in Table 2.

Table 2. Summary of study characteristics of the 22 studies included in the meta-analyses.

Study characteristic	Sub-groups	Number of studies	
Model of experimental arthritis	CIA	16	
	AIA	8	
Species	Rat	16	
	Mice	6	
Drugs tested	synthetic DMARDs	Dose mg/kg	
	Methotrexate	*10*	*0.1-50mg/kg*
	Leflunomide	*2*	*3.75-10mg/kg*
	Cyclosporine	*1*	*2.5mg/kg*
	Sulfasalazine	*1*	*80mg/kg*
	Azathioprine	*1*	*5mg/kg*
	Hydroxychloroquine	*1*	*25mg/kg*
	Methylprednisolone	*1*	*2mg/kg*
	Dexamethasone	*1*	0.5mg/mg
	Biological DMARDs		Dose mg/kg
	anti-TNF	*5*	*0.75-2mg/kg*
	Anti-IL-1	*4*	*0.1-1mg/kg*
	CTLA4-Ig	*2*	*1-5mg/kg*
Route of administration	Oral	15	
	Subcutaneous	6	
	Intraperitoneal	9	

Treatment duration	1 week	6
	2-4 weeks	8
	>1 month	8
Treatment strategy	Prophylactic	16
	Pre-arthritis	9
	Therapeutic	12
Clinical outcome	Arthritis severity score	14
	Paw swelling (mm)	8
	Paw volume (ml)	5
Joint structural changes	(Semi-)quantitative histological data scores:	
	synovial hyperplasia	*5*
	cell infiltration	*10*
	pannus formation	*3*
	oedema	*1*
	fibrosis	*2*
	cartilage destruction	*10*
	bone erosion	*10*
	Quantitative radiographic scores based on:	
	X-ray	8
	microCT	2

CIA = collagen induced arthritis, AIA = adjuvant induced arthritis, DMARDs = disease modifying anti-rheumatic drugs.

Data extraction

We extracted individual study characteristics from each publication, and, where a publication reported more than 1 experiment, these data were also extracted and considered independent experiments. Extracted data included: experimental arthritis model, species, number of animals per group, drug and dose, route and time of drug administration, clinical-, histological- or radiological scores. Where arthritis severity measurements were performed serially, we only extracted the final time point.

Quality assessment of methodology

Study quality and risk of selection- and detection bias was assessed by a modified 11-point-item checklist, adapted from CAMARADES (Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental studies) [13]. The checklist comprises items of study methodology: randomisation (1); allocation concealment (2); blinding (3); evidence of induced arthritis (macroscopic, histological or radiological) (4), sample size/power calculations (5), statement of conflict of interest (6), statement of compliance with animal welfare regulations (7), standardized method for data collection (8), (semi)quantitative scoring method for disease activity (9), (semi)quantitative scoring method for joint damage (10) and clear data presentation (11). Each item was scored as 1 if the data were reported satisfactorily and 0 if not (unclear risk of bias) and maximum score was 11. The median quality score of six was considered to be sufficiently high quality for further analysis. An overview of the checklist is depicted in table 3**.**

Table 3. Methodological quality assessment form for study quality and potential risk of bias as judged by the quality of reporting. Each item was scored as 1 if performed and 0 if not reported or not performed. Maximum score was 11 points.

Risk of Bias	Criteria		Explanation
Selection bias	1.	Randomization	
	2.	Allocation concealment	Concealing the allocation sequence from those assigning animals to experimental and control groups until moment of assessment.
Detection bias	3.	Blinding	Keeping the persons who perform the experiment, collect data and assess outcome unaware of the treatment allocation
Other sources of bias	4.	Evidence of proper arthritis induction	Histological, macroscopic, microscopic or X-ray evidence
	5.	Sample size/power calculations before start of experiment	
	6.	Statement regarding potential conflict of interest	
	7.	Statement of compliance with animal welfare regulations	
	8.	Standardized method data collection	Data collection at predefined time points
	9.	Validated scoring method for arthritis severity	Semi-quantitative clinical scoring system for each paw in a range from 0-4, caliper measurements of ankle joints, or use of a plethysmometer for measurements of small volume changes in paw volume.
	10.	Validated scoring method for joint damage	X-ray: modified Larsen scoring method. Histology: semi-quantitative for synovial and extra articular inflammation (in a range from 0-3) and bone erosions (in a range from 0-5).
	11.	Clear data presentation	Numbers of animals per group, data of both treatment and control group available.

Categorisation of studies according to type of intervention

Studies were discerned into three groups according to the time point of treatment initiation: (1) Prophylactic treatment (day 0-9): therapy is initiated prior to injection of arthritis-stimulators or after injection of arthritis-stimulator but prior to the development of a systemic autoimmune response which is characterized by production of autoantibodies. (2) Pre-arthritis treatment (day 10-20): start of therapy after the development of auto-immunity but before the onset of clinically evident arthritis. (3) Therapeutic treatment (>20 day): initiation of treatment after the onset of arthritis, which occurs generally around day 21-22.

Meta-analysis

Only studies with high methodological quality (score ≥ 6) were summarized in meta-analyses. Furthermore; studies that did not provide standard deviations (SD) or standard errors of the mean (SEM) were excluded for the meta-analyses. To compare the severity of arthritis in the treatment group to an arthritic untreated control animal, we calculated the normalized mean difference (NMD) of arthritis clinical score, paw volume or paw swelling. The NMD effect size in the treated animals is calculated as a proportion of the mean in the control group [14]. Similarly, we calculated the NMD for each experimental comparison as the proportional improvement in the treated group compared with the control group, along with the standard error of the estimate. To account for anticipated heterogeneity we applied the DerSimonian and Laird random effects model [15] to calculate an overall treatment effect, this random-effects model for meta-analysis uses both within-the study variance and the between-study variance. We used Cochran's Q to calculate heterogeneity of the studies [16].

Results

Study selection and study quality

Our systematic search identified a total of 3486 titles from eight databases (Figure 2), 35 studies met the inclusion criteria based on full paper assessment. A summary overview of these 35 studies is presented in Table 1, for the complete overview see online supplementary Table S5. For further selection of studies for meta-analysis, 28 studies were considered to be of sufficient high methodological quality (score ≥ 6). Six of these studies did not report SDs or SEMs and therefore and could not be included in the meta-analyses [17-22]. Finally, the results of 22 studies, using a total of 712 animals (control animals, n =263 and treated animals, n = 449), were summarized in meta-analyses.

The most frequently used model of experimental arthritis was the CIA model (CIA 16 studies, AIA 8 studies). 16 studies used rats and 6 studies used mice (table 2). Thirteen out of 22 publications (59%) reported the blinded assessment of outcome, 15 (68%) reported randomization, and none reported performing a sample size calculation (table 4**)**. The outcome summarized in the meta-analysis was the proportion reduction of arthritis severity (expressed in NMD).

Histological- and radiographic scores were not consistently scored in uniform scoring scales. Therefore it was impossible to perform meta-analyses on these outcomes.

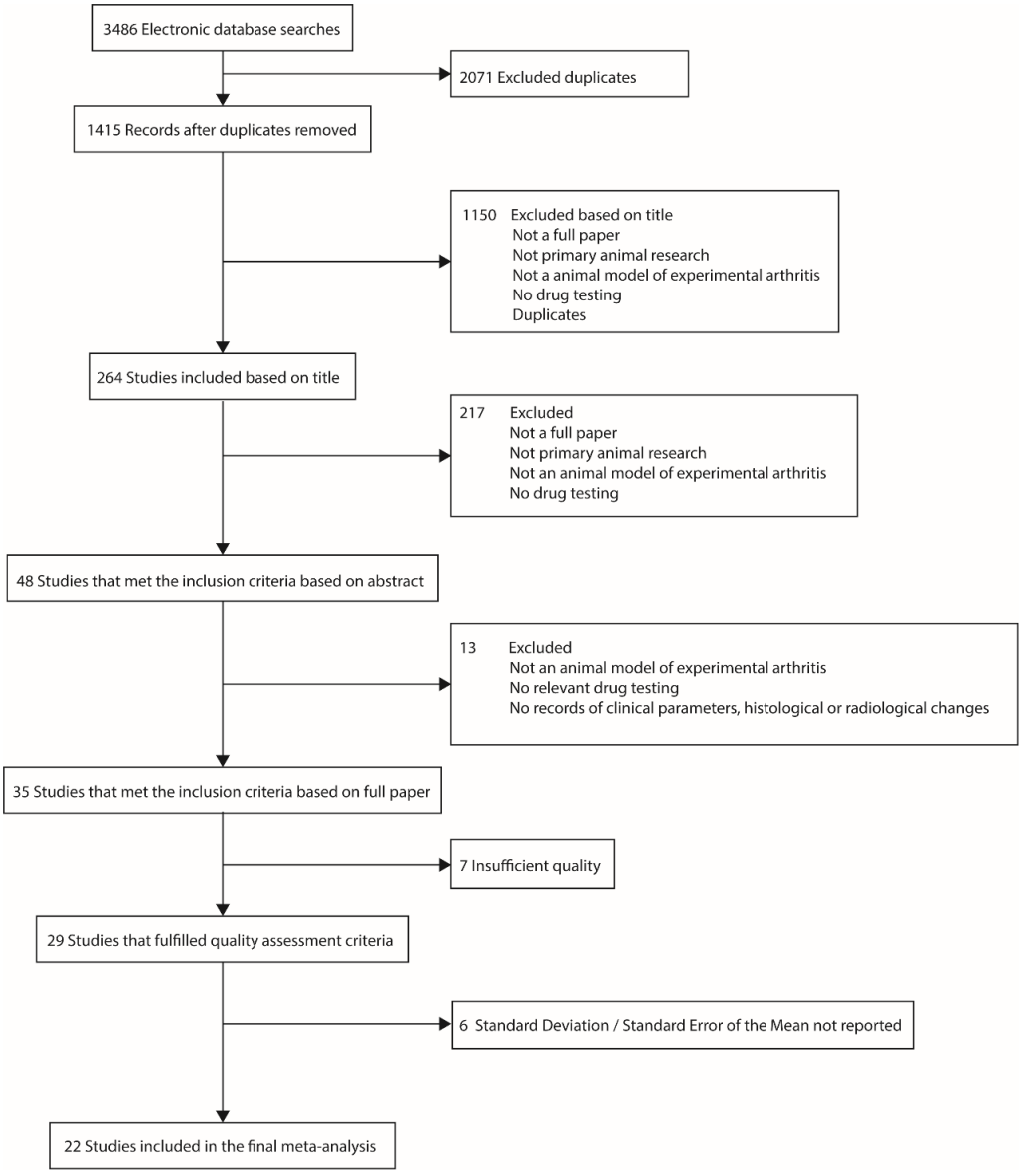


Figure 2. Flow diagram of study selection.

Table 4. Number and percentage of publications reporting individual components of the study quality checklist for the 22 studies that were included in the meta-analyses.
The quality checklist is depicted in table 3.

Quality criteria	Total studies (n=22)	%
Randomisation	15	69
Allocation concealment	9	40
Blinding	13	59
Evidence of proper arthritis induction	20	90
Sample size/power calculations	0	0
Statement regarding potential conflict of interest	7	32
Statement of compliance with animal welfare	17	77
Standardized method for data collection	18	81
Validated scoring method for arthritis severity	21	100
Validated scoring method for joint damage	19	86
Clear data presentation (group size, treatment and control groups)	14	63

Table 5. List of all 35 studies that met the inclusion criteria based on full paper assessment. Twenty-two studies [20-41] are included in the meta-analyses.

Author	Type of intervention	Model	Species	DMARD tested	Treatment duration	Outcome parameters	Quality score
Morgan 2001 [23]	prophylactic	AIA	rats	Methotrexate	6 weeks	paw swelling (mm) clinical score (0-16)	9
Lee 2009 [24]	prophylactic	CIA	mice	Methotrexate	3 weeks	clinical score (0-16), incidence (%)	8
Rovensky 2009 [25]	prophylactic	AIA	rats	Methotrexate	7 weeks	paw volume (ml)	7
Rovensky 2003 [26]	prophylactic	AIA	rats	Cyclosporin A Methotrexate	7 weeks 7 weeks	paw swelling (mm)	6
Smith 1996 [27]	prophylactic	CIA	rats	Methylpredni-solone Methotrexate Azathioprine Hydroxy-chloroquine	4 weeks 4 weeks 4 weeks 4 weeks	clinical score (0-16), Δ paw volume	7
Al-Abd AM 2014 [28]	prophylactic	CIA	mice	Leflunomide	5 weeks	clinical score (0-16)	6
Zuurmond 2011 [29]	prophylactic	AIA	rats	Anti-IL-1 Dexametha-sone	2 weeks 2 weeks	clinical score (0-16), paw swelling (mm)	8
Webb 1996 [30]	prophylactic, therapeutic	CIA	mice	CTLA4-Ig	2 weeks	paw swelling (mm), clinical score (0-12)	7
Knoerzer 1995 [31]	prophylactic	CIA	rats	CTLA4-Ig	2 weeks	clinical score (0-16)	8
Gowayed 2015 [32]	pre-arthritis	AIA	rats	Leflunomide	2 weeks	paw swelling (mm)	8
Sakuma 2001 [33]	pre-arthritis	AIA	rats	Methotrexate	1 week	paw volume (ml)	7

Le 2009 [34]	pre-arthritis	CIA	rats	Methotrexate	once	clinical score (0-8), paw swelling (mm)	6
Du 2008 [35]	pre-arthritis	CIA	rats	Methotrexate	3 weeks	clinical score (0-8)	7
Setoguchi 2010 [36]	pre-arthritis	CIA	rats	Etanercept	1 week	paw volume (ml)	7
Bendele 2000 [37]	pre-arthritis	CIA AIA	rats	Anti-IL-1	1 week	paw volume (ml)	7
Fener 1990 [38]	therapeutic	CIA	rats	Sulfazalazine	17 weeks	clinical score (0-12)	8
Zhang 2013 [39]	therapeutic	CIA	mice	Methotrexate Etanercept	6 weeks 6 weeks	clinical score (0-16)	6
Saadat 2005 [40]	therapeutic	CIA	rats	Methotrexate	2 weeks	clinical score (0-16)	9
O'Valle 2015 [41]	therapeutic	CIA	mice	Etanercept	4 weeks	paw swelling (mm), clinical score (0-16)	8
Joosten 1996 [42]	therapeutic	CIA	mice	Anti-IL1 Etanercept	1 week 1 week	clinical score (0-8)	6
Yang 2010 [43]	therapeutic	CIA	rats	Etanercept	2 weeks	paw swelling (mm)	8
Bendele 1999 [44]	therapeutic, pre-arthritis	CIA, AIA	rats	Anti-IL-1	1 week	paw volume (ml)	6
Yi 2014 [17]	prophylactic	CIA	mice	Etanercept	5 weeks	clinical score (0-16) and incidence (%)	6
Chen 2012 [18]	therapeutic	CIA	rats	Metrotrexate	4 weeks	clinical score (0-16)	7
Xinqiang 2010 [19]	therapeutic	CIA	rats	Metrotrexate	4 weeks	clinical score (0-16)	7
Kliwinski 2005 [20]	prophylactic	CIA	rats	CTLA4-Ig	1 week	paw volume (ml)	6

Hsu 2010 [21]	pre-arthritis	CIA	rats	Etanercept	4 weeks	clinical score (0-8), paw swelling (mm)	7
Stolina 2009 [22]	prophylactic	CIA, AIA	rats	Anti-IL-1	1 week	paw volume (ml), paw swelling (mm)	8
Kim YH 2015 [45]	pre-arthritis	CIA	rats	Methotrexate	1 week	only histology and radiological outcome	5
Yao 2013 [46]	therapeutic	CIA	rats	Leflunomide, Metrotrexate	3 weeks 3 weeks	clinical score (0-16)	5
Teramachi 2011 [47]	prophylactic	AIA	rats	Metrotrexate	3 weeks	paw volume (ml)	4
Baggott 2007 [48]	prophylactic	AIA	rats	Metrotrexate	4 weeks	only radiological outcome	5
Brauer 1994 [49]	prophylactic	AIA	rats	Cyclosporin A	2-4 weeks	paw swelling (mm)	2
Wooley 1993 [50]	pre-arthritis	CIA	mice	Anti-IL-1	2 weeks	clinical score (0-12), paw swelling (mm)	5
Brahn 1991 [51]	prophylactic	CIA	rats	Cyclosporin A Methotrexate	3 weeks 3 weeks	clinical score (0-16) and incidence (%)	5

Effect of intervention on clinical outcome

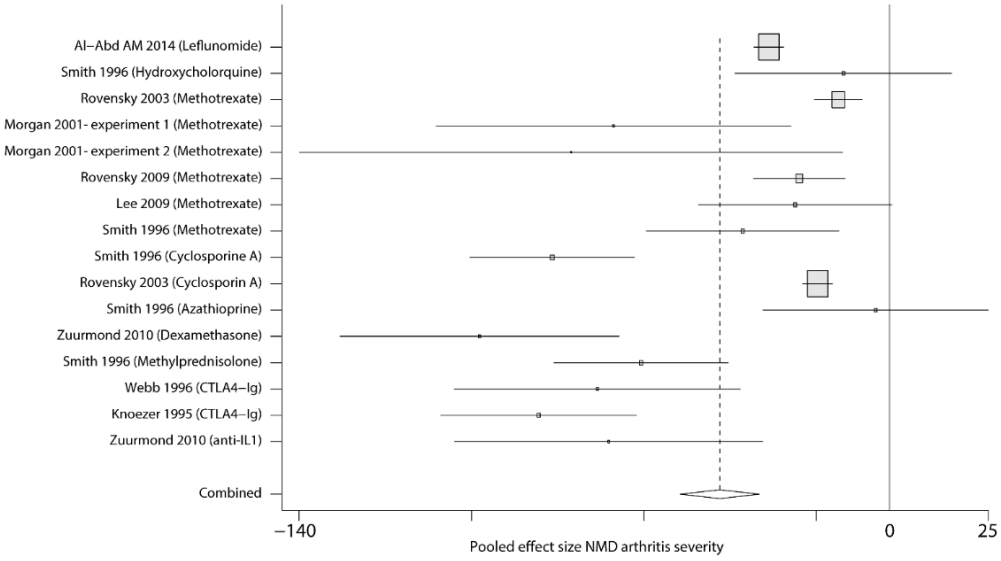
The treatment efficacy on arthritis severity was studied per disease stage.

Prophylactic treatment

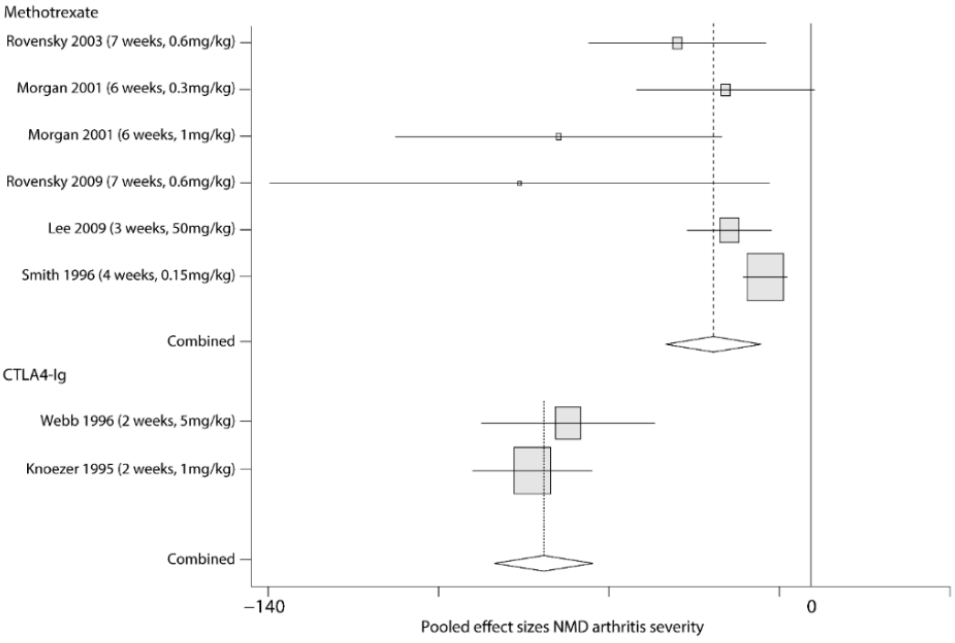
Sixteen experiments (including 312 animals) studied the effect of prophylactic intervention on the severity of arthritis (Figure 3A). Several DMARDs were tested in a prophylactic setting (*methotrexate* [6 experiments] [23-27]*, leflunomide* [1 experiment] [28]*, cyclosporine A* [2 experiments] [26 27]*, azathioprine* [1 experiment] [27]*, hydroxycholoroquine* [1 experiment] [27]*,* and *methylprednisolone/dexamethasone* [2 experiments] [27 29], *anti-IL1* [1 experiments] [29] and *CTLA4-Ig* [2 experiments] [30 31]. The combined effect size of the different studies indicated that prophylactic intervention is associated with a reduction of arthritis severity in animal models of arthritis ($p<0.001$). Prophylactic treatment with both methotrexate ($p< 0.001$) and CTLA4-Ig ($p<0.001$) was significantly associated with a reduction in arthritis severity (Figure 3B).

Figure 3. Prophylactic intervention in experimental models of arthritis. Effect of prophylactic intervention on arthritis severity reported in 16 individual experiments using synthetic or biological DMARDs. The pooled effect size of normalized mean difference (NMD) in arthritis severity is -40.1 (95CI=-50.6 to -31.4, z-value=-8.2, $p<0.001$) (A). Stratified meta-analysis of the different experiments investigating the effects of different anti-rheumatic drugs further specified for methotrexate, pooled estimate -26.0 (95CI=-38.6 to -13.5, $p<0.001$) and CTLA4-Ig, pooled estimate -80.2 (95CI=-100.0 to -60.7, $p<0.001$). Treatment length was on average 5.5 weeks for methotrexate and 2 weeks for CTLA4-Ig (B).

A Prophylactic treatment with DMARDs



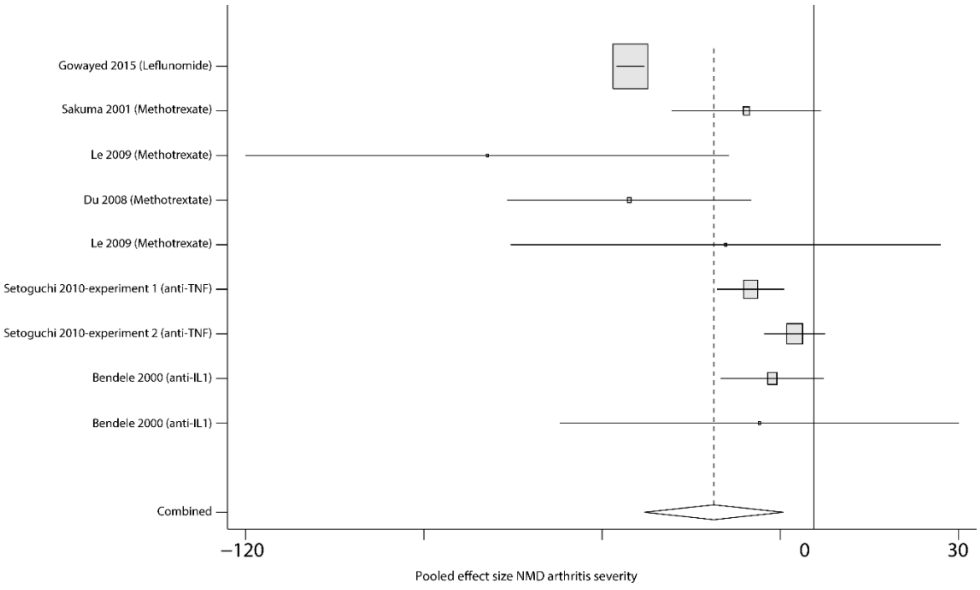
B Prophylactic treatment stratified for DMARD



Pre-arthritis treatment

Nine experiments (including 156 animals) studied the effect of early pre-arthritis treatment on arthritis severity (Figure 4A). DMARDs tested were *leflunomide* [1 experiments] [32], *methotrexate* [4 experiments] [33-35]*, anti-TNF* [2 experiments] [36] *, anti-IL1* [2 experiments] [37]. A meta-analysis of these studies demonstrated that pre-arthritis intervention is associated with a reduction of arthritis severity in animal models of arthritis (p=0.005). Stratified analysis for most commonly used DMARDs revealed that treatment with methotrexate was significantly associated with less arthritis severity (p<0.01), while no statistically significant results were obtained for anti-TNF (p=0.065) and anti-IL1 (p=0.098) (Figure 4B). None of the animal studies performed in a pre-arthritis stage evaluated a reduction of arthritis incidence or a delay in arthritis onset after short-term treatment.

A Pre-arthritis treatment with DMARDs



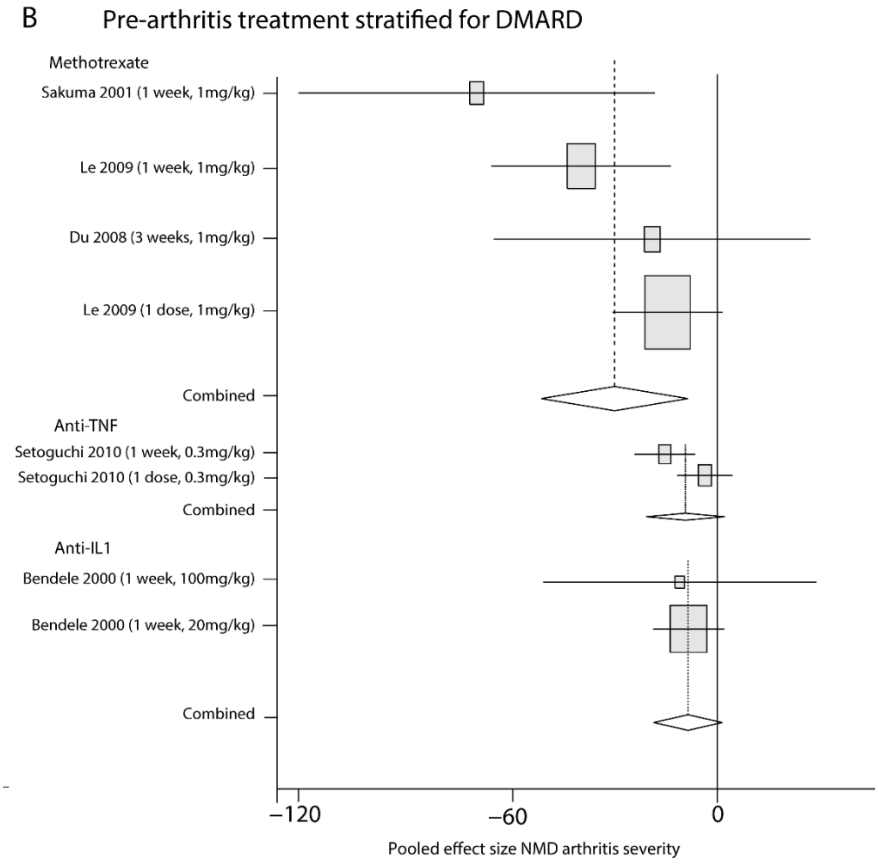
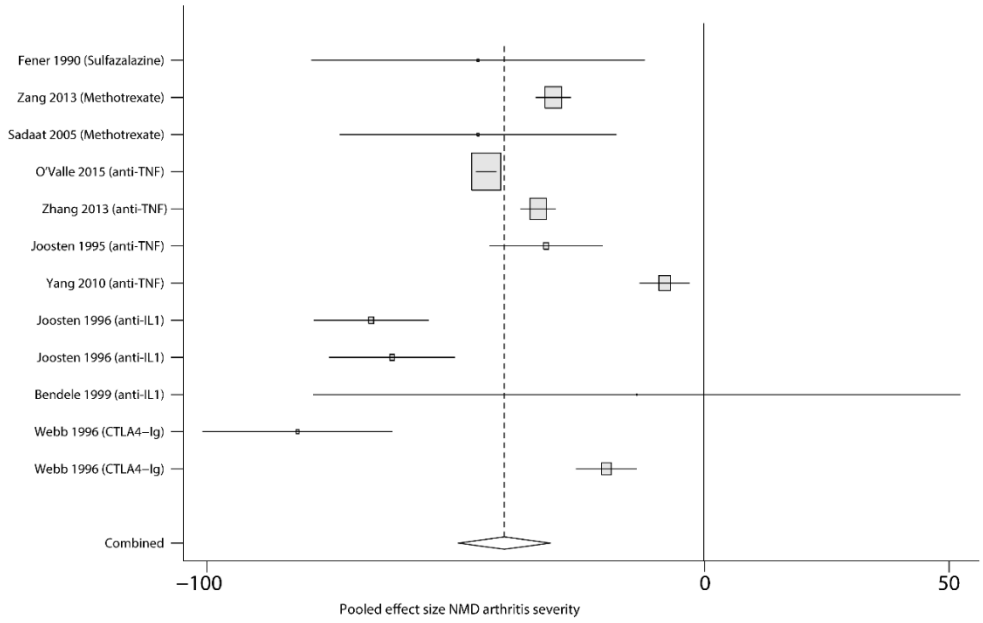


Figure 4. Pre-arthritis intervention in experimental models of arthritis. Effect of pre-arthritis intervention on arthritis severity reported in 9 individual experiments using synthetic or biological DMARDs in a pre-arthritis phase of arthritis. The pooled effect size of normalized mean difference (NMD) in arthritis severity is -21.2 (95CI=-35.9 to -6.5, z-value=-2.8, p=0.005) (A). Stratified meta-analysis of the different experiments investigating the effects of different anti-rheumatic drugs further specified for methotrexate pooled estimate -29.5 (95CI=-50.6 to -8.4, p=0.006), anti-TNF pooled estimate -8.6 (95CI=-17.7 to 0.5, p=0.065), and anti-IL1 pooled estimate -9.0 (95CI=-19.6 to 1.6, p=0.098). Treatment length was on average 1 week for methotrexate, anti-TNF and anti-IL1 (B).

Therapeutic treatment

Twelve experiments (including 244 animals) studied the effect of therapeutic treatment in established arthritis (Figure 5A). Anti-rheumatic drugs tested in established disease were *sulfasalazine* [1 experiment] [38], *methotrexate* [2 experiments] [39 40] *, anti-TNF* [4 experiments] [39 41-43]*, anti-IL1* [3 experiments] [42 44] and *CTLA4-Ig* [2 experiments] [30]. Therapeutic treatment with methotrexate ($p<0.001$), anti-TNF ($p<0.001$) anti-IL1 ($p<0.001$) and CTLA4-Ig ($p<0.001$) in established disease were all significantly associated with a reduced arthritis severity (Figure 5B).

A Therapeutic intervention with DMARDs in established disease



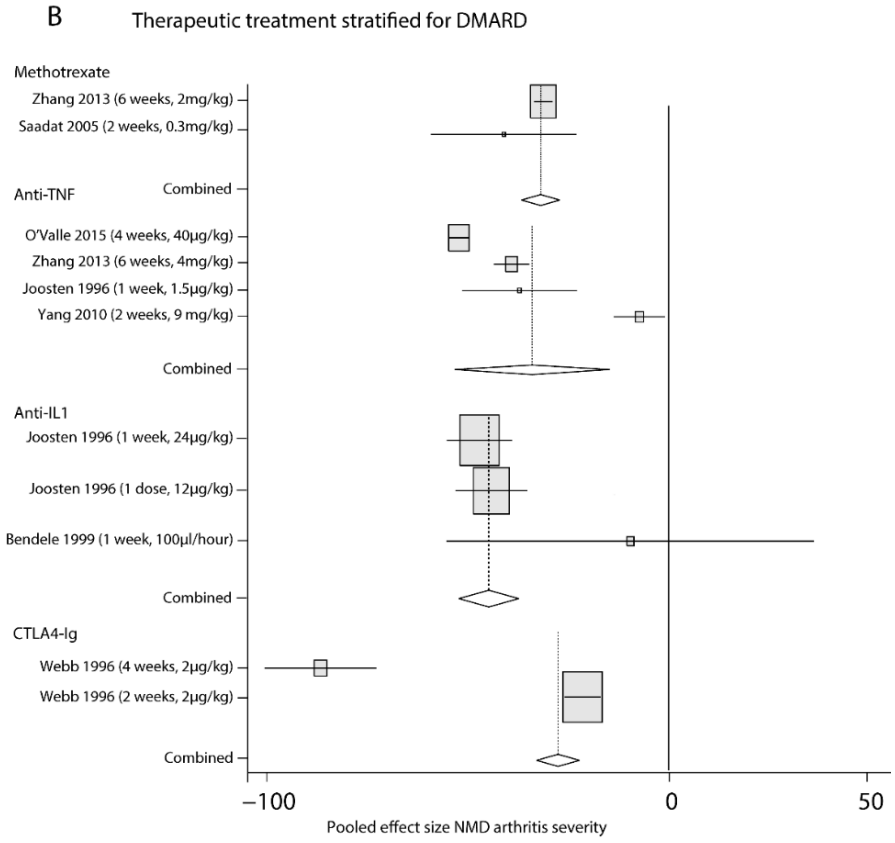


Figure 5. Therapeutic intervention in experimental models of arthritis. Effect of therapeutic intervention on arthritis severity reported in 12 individual experiments using synthetic or biological DMARDs in established arthritis. The pooled effect size of normalized mean difference (NMD) in arthritis severity is -44.2 (95CI=-54.4 to -34.0, z-value=-8.5, p<0.001) (A). Stratified meta-analysis of the different experiments investigating the effects of different anti-rheumatic drugs further specified for methotrexate -34.4 (95CI=-42.5 to -26.3, p<0.001), anti-TNF -32.2 (95CI=-49.2 to -15.1, p<0.001), anti-IL1 -70.6 (95CI=-79.9 to -61.3, p<0.001)and CTLA4-Ig -27.9 (95CI=-34.3 to -21.5, p<0.001). Treatment length was on average 4 weeks for methotrexate, 3.3 weeks for anti-TNF, 1 week for anti-IL1 and 3 weeks for CTLA4-Ig (B).

Sub-analyses

Since CIA and AIA somewhat differ in disease pathology we have performed separate sub-analyses for these models which showed similar results as that of the total group (Fig. 6, 7).

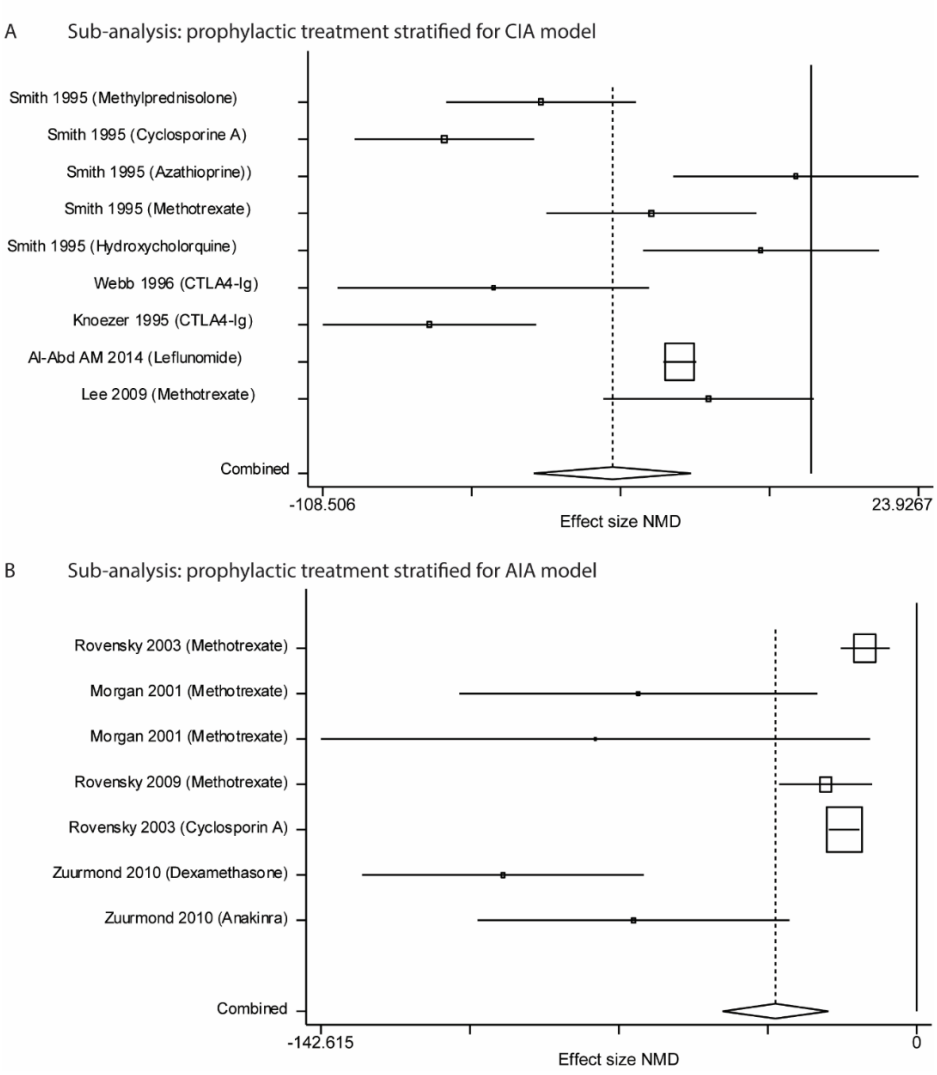


Figure 6. Sub-analysis of prophylactic intervention stratified for CIA and AIA. Effect of prophylactic intervention on arthritis severity stratified for the CIA model (9 studies). The pooled effect size of normalized mean difference (NMD) in arthritis severity is -44.1 (95CI=-61.6 to -26.6, z-value=-4.9, p<0.001) (A). Stratified meta-analysis of the different experiments investigating the effects of different anti-rheumatic drugs further specified for the AIA model (7 studies), pooled estimate -33.9 (95CI=-46.6 to -21.2, z-value = -5.2, p<0.001) (B).

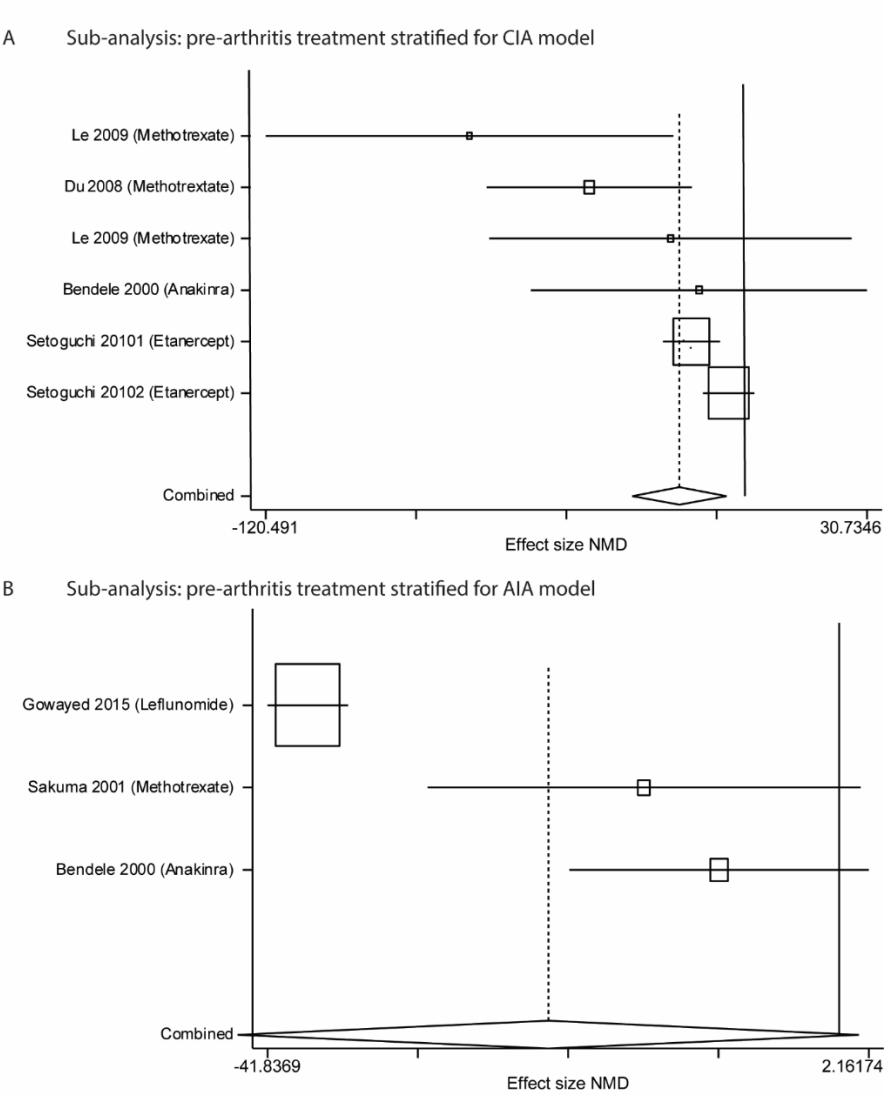


Figure 7. Sub-analysis of pre-arthritis intervention stratified for CIA and AIA. Effect of prophylactic intervention on arthritis severity stratified for the CIA model (6 studies). The pooled effect size of normalized mean difference (NMD) in arthritis severity is -16.5 (95CI=-16.5 to -28.3, z-value=-4.6, p=0.006) (A). Stratified meta-analysis of the different experiments investigating the effects of different anti-rheumatic drugs further specified for the AIA model (3 studies), pooled estimate -21.3 (95CI=-44.0 to 1.5, p=0.07).

Discussion

The present systematic literature review and the meta-analyses of data on treatment in animal models of experimental arthritis reveal that 'prophylactic and pre-arthritis treatment strategies' are effective and result in less severe disease. Currently research emphasises on early identification of individuals at risk for developing RA with as ultimate goal the reduction of disease severity or even to prevent clinically manifest disease. Although it will take years before the results of current clinical trials will be at the stage of publishing, results of the present study in animals support the concept that very early treatment may be effective. Observational studies in RA patients have provided evidence that support the concept of a therapeutic 'window of opportunity'. The period in which the disease is most susceptible to treatment is presumed to consist of the first three months after symptom onset [52 53]. Although some studies have treated patients rapidly after arthritis has become clinically evident [54-57], studies that initiate treatment already in pre-arthritis phases are more challenging to perform, because of the difficulty to identify patients with arthralgia and a high predicted risk for developing RA. Some of the preventive studies in human RA aim to target autoantibody positive subjects with arthralgia and we hypothesize that this phase corresponds to intervention in the pre-arthritis phase of the CIA model. But, although the CIA mouse model is widely used to mimic the antibody-dependent process of RA pathogenesis, anti-collagen antibodies are not the most prominent antibodies in human RA and conflicting results on ACPA induction in CIA exist [58]. Thus, while CIA and AIA are informative, these models only partially resemble the situation in human RA and reflect only some of the basic disease mechanisms and molecular pathways involved in RA development. Despite the disparities between animal models of arthritis and RA, animal models of arthritis are of interest, and the summarized data suggest that very early treatment is effective.

The second aim was to evaluate the effect of different medications in animal models. The most frequently studied DMARDs were methotrexate and anti-TNF. In humans several other drugs (abatacept, hydroxychloroquine, atorvastatin) are now also being investigate in pre-arthritis phases. Studies investigating the effect of hydroxychloroquine on animal models are limited; in our meta-analysis we included only one study that evaluated hydroxychloroquine on CIA. Our literature search was limited to studies that tested DMARDs, statins were not included. Nevertheless, the effects of statins have been studied, with contrasting results; some studies reported anti-inflammatory effects [44, 45] while others pointed to an accelerated onset of CIA in mice [46]. In our meta-analysis we observed a higher effect for metrotrexate then for anti-TNF, this may suggest that methotrexate is more disease-modifying in this very early disease phase. Though, formal conclusions on the difference in efficacy or treatment dose cannot be made and translation to the human setting is limited. None of the pre-arthritis studies compared the different medications

head-to-head. We distinguished treatment started in the initiation phase (prophylactic intervention) and in the phase autoantibodies had developed (pre-arthritis intervention). Meta-analyses suggested that treatment started in both phases was effective. However, none of the studies performed side-by-side comparisons of prophylactic, pre-arthritis and therapeutic interventions. Therefore we cannot conclude if the first two strategies are more effective than treatment initiated in the established disease phase. Similarly, the results obtained on prophylactic strategies cannot be compared to those of pre-arthritis strategies. There are more limitations. Surprisingly, the majority of studies that aimed to test the efficacy of prophylactic treatment used an extended treatment period (average of 4.6 weeks, thus continuing within the phase of established disease). Principally, treatment is preventive if given in pre-clinical phases only. Studies that initiated treatment at a pre-arthritis stage did treat animals with a short course of (at average) 1 week and still observed less severe arthritis in the clinical phase of the disease [33-37]. Furthermore none of the studies evaluated the occurrence of clinical arthritis. Hence there are no data from animal models to conclude if clinical disease can be prevented. For human translation, it would be most interesting to determine whether preventive treatment can actually reverse autoimmunity and prevent RA.

The studies that were evaluated were heterogeneous in several aspects. The use of standard operating procedures for validation of results is crucial to reduce study heterogeneity. Using a 11-point-item quality checklist we aimed to select studies that had a reliable study design; despite the funnel used, still considerable differences were present in the experiments that were included in the meta-analysis. Mice as well as rats were studied. In addition, results of two different animal models (CIA, AIA) were evaluated. Sub-analyses stratified for CIA and AIA, however, showed similar results as that of the total group. Furthermore, arthritis severity was assessed in different ways. Although a validated method was use to compared these outcomes (normalized mean difference), this adds to the heterogeneity. For these different reasons, the NMDs –including those resulting from the meta-analyses performed for different disease phases and for the different treatments- should not be compared in their effect size. Thus although the efficacy of different treatment strategies cannot be compared, this study provides an overview of all available data on animal models and provides an evaluation if treatment initiated in very early disease phases is effective.

Based on the present evaluation of the available literature we conclude that the ideal experiment on animals for this research question should still be performed. This study should test interventions side-by-side in different disease phases and with a similar treatment schedule to be able to compare efficacy. In addition, a head-to-head comparison of DMARDs like methotrexate, abatacept, rituximab and anti-TNF with a defined duration could answer the question whether pre-arthritis treatment can reverse autoimmunity and prevent arthritis in mouse models. Proper controls are sham-treated mice that develop a

natural course of experimental-induced arthritis. The presence and evolution of systemic autoimmunity in CIA, defined by autoantibody production against CII, should be determined and linked to clinical outcome. Ideally, the effect of (a short course of) pre-arthritis treatment should be evaluated over an extended period of time to determine whether there is long-term arthritis-free-'benefit' or a delayed onset of arthritis.

In conclusion, this study systematically evaluated the results of animal studies and suggested that both prophylactic and pre-arthritis treatment strategies lead to a significant reduction of arthritis severity scores and hits at a possibility for preventive therapy in RA. However, larger studies are needed to confirm this.

References

1. van der Linden MP, le Cessie S, Raza K, et al. Long-term impact of delay in assessment of patients with early arthritis. Arthritis and rheumatism 2010;**62**(12):3537-46.
2. Anderson JJ, Wells G, Verhoeven AC, et al. Factors predicting response to treatment in rheumatoid arthritis: the importance of disease duration. Arthritis and rheumatism 2000;**43**(1):22-9 .
3. Feldman DE, Bernatsky S, Houde M, et al. Early consultation with a rheumatologist for RA: does it reduce subsequent use of orthopaedic surgery? Rheumatology 2013;**52**(3):452-9.
4. Symmons DP, Jones MA, Scott DL, et al. Longterm mortality outcome in patients with rheumatoid arthritis: early presenters continue to do well. The Journal of rheumatology 1998;**25**(6):1072-7
5. van Vollenhoven RF, Nagy G, Tak PP. Early start and stop of biologics: has the time come? BMC medicine 2014;**12**:25.
6. Knip M, Virtanen SM, Seppa K, et al. Dietary intervention in infancy and later signs of beta-cell autoimmunity. The New England journal of medicine 2010;**363**(20):1900-8.
7. Keymeulen B, Vandemeulebroucke E, Ziegler AG, et al. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. The New England journal of medicine 2005;**352**(25):2598-608.
8. Orban T, Bundy B, Becker DJ, et al. Costimulation modulation with abatacept in patients with recent-onset type 1 diabetes: follow-up 1 year after cessation of treatment. Diabetes care 2014;**37**(4):1069-75.
9. Holmdahl R, Andersson M, Goldschmidt TJ, et al. Type II collagen autoimmunity in animals and provocations leading to arthritis. Immunological reviews 1990;**118**:193-232
10. Pearson CM. Development of arthritis, periarthritis and periostitis in rats given adjuvants. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine 1956;**91**(1):95-101
11. Batsalova T, Lindh I, Backlund J, et al. Comparative analysis of collagen type II-specific immune responses during development of collagen-induced arthritis in two B10 mouse strains. Arthritis research & therapy 2012;**14**(6):R237.
12. Kim EY, Chi HH, Bouziane M, et al. Regulation of autoimmune arthritis by the pro-inflammatory cytokine interferon-gamma. Clinical immunology 2008;**127**(1):98-106.
13. Macleod MR, O'Collins T, Howells DW, et al. Pooling of animal experimental data reveals influence of study design and publication bias. Stroke; a journal of cerebral circulation 2004;**35**(5):1203-8.
14. Vesterinen HM, Sena ES, Egan KJ, et al. Meta-analysis of data from animal studies: a practical guide. Journal of neuroscience methods 2014;**221**:92-102 doi: 10.1016/j.jneumeth.2013.09.010[published Online First: Epub Date]|.
15. DerSimonian R, Laird N. Meta-analysis in clinical trials. Controlled clinical trials 1986;**7**(3):177-88
16. Cochran WG. The Combination of Estimates from Different Experiments. Biometrics 1954;**10**(1):101-29.

17. Yi H, Kim J, Jung H, et al. Induced production of anti-etanercept antibody in collagen-induced arthritis. Mol Med Rep 2014;**9**(6):2301-08.
18. Chen Z, Tu S, Hu Y, et al. Prediction of response of collagen-induced arthritis rats to methotrexate: an (1)H-NMR-based urine metabolomic analysis. J Huazhong Univ Sci Technolog Med Sci 2012;**32**(3):438-43.
19. Xinqiang S, Fei L, Nan L, et al. Therapeutic efficacy of experimental rheumatoid arthritis with low-dose methotrexate by increasing partially CD4+CD25+ Treg cells and inducing Th1 to Th2 shift in both cells and cytokines. Biomed Pharmacother 2010;**64**(7):463-71.
20. Kliwinski C, Kukral D, Postelnek J, et al. Prophylactic administration of abatacept prevents disease and bone destruction in a rat model of collagen-induced arthritis. J Autoimmun 2005;**25**(3):165-71.
21. Hsu YH, Chang MS. Interleukin-20 antibody is a potential therapeutic agent for experimental arthritis. Arthritis and rheumatism 2010;**62**(11):3311-21.
22. Stolina M, Schett G, Dwyer D, et al. RANKL inhibition by osteoprotegerin prevents bone loss without affecting local or systemic inflammation parameters in two rat arthritis models: comparison with anti-TNFalpha or anti-IL-1 therapies. Arthritis research & therapy 2009;**11**(6):R187.
23. Morgan SL, Baggott JE, Bernreuter WK, et al. MTX affects inflammation and tissue destruction differently in the rat AA model. The Journal of rheumatology 2001;**28**(7):1476-81
24. Lee JD, Huh JE, Baek YH, et al. The efficacy and mechanism action of RvCSd, a new herbal agent, on immune suppression and cartilage protection in a mouse model of rheumatoid arthritis. J Pharmacol Sci 2009;**109**(2):211-21.
25. Rovensky J, Stancikova M, Rovenska E, et al. Treatment of rat adjuvant arthritis with flavonoid (Detralex), methotrexate, and their combination. Ann N Y Acad Sci 2009;**1173**:798-804.
26. Rovensky J, Svik K, Stancikova M, et al. Effect of immunostimulatory ribomunyl on the preventive treatment of rat adjuvant arthritis with cyclosporine and methotrexate. The Journal of rheumatology 2003;**30**(9):2027-32.
27. Smith RJ, Sly LM. Type II collagen-induced arthritis in the diabetic-resistant biobreeding rat: inflammatory and histopathological features of joint pathology and effects of antiinflammatory and antirheumatic drugs on this chronic arthritic process. J Pharmacol Exp Ther 1996;**277**(3):1801-13
28. Al-Abd AM, Al-Abbasi FA, Nofal SM, et al. Nimesulide improves the symptomatic and disease modifying effects of leflunomide in collagen induced arthritis. PLoS One 2014;**9**(11):e111843.
29. Zuurmond AM, Koudijs A, van EB, et al. Integration of efficacy, pharmacokinetic and safety assessment of interleukin-1 receptor antagonist in a preclinical model of arthritis. Regul Toxicol Pharmacol 2011;**59**(3):461-70.
30. Webb LM, Walmsley MJ, Feldmann M. Prevention and amelioration of collagen-induced arthritis by blockade of the CD28 co-stimulatory pathway: requirement for both B7-1 and B7-2. Eur J Immunol 1996;**26**(10):2320-28.

31. Knoerzer DB, Karr RW, Schwartz BD, et al. Collagen-induced arthritis in the BB rat. Prevention of disease by treatment with CTLA-4-Ig. J Clin Invest 1995;**96**(2):987-93.
32. Gowayed MA, Refaat R, Ahmed WM, et al. Effect of galantamine on adjuvant-induced arthritis in Rats. Eur J Pharmacol 2015.
33. Sakuma S, Nishigaki F, Magari K, et al. FK506 is superior to methotrexate in therapeutic effects on advanced stage of rat adjuvant-induced arthritis. Inflamm Res 2001;**50**(10):509-14
34. Le GB, Soltner E, Charrier C, et al. A combination of methotrexate and zoledronic acid prevents bone erosions and systemic bone mass loss in collagen induced arthritis. Arthritis research & therapy 2009;**11**(6):R185.
35. Du F, Lu LJ, Fu Q, et al. T-614, a novel immunomodulator, attenuates joint inflammation and articular damage in collagen-induced arthritis. Arthritis research & therapy 2008;**10**(6):R136.
36. Setoguchi C, Tsuji F, Katsuta O, et al. Combined effects of bucillamine and etanercept on a rat type II collagen-induced arthritis model. Mod Rheumatol 2010;**20**(4):381-88.
37. Bendele AM, Chlipala ES, Scherrer J, et al. Combination benefit of treatment with the cytokine inhibitors interleukin-1 receptor antagonist and PEGylated soluble tumor necrosis factor receptor type I in animal models of rheumatoid arthritis. Arthritis and rheumatism 2000;**43**(12):2648-59.
38. Fener P, Bannwarth B, Gillet P, et al. Influence of sulfasalazine on established collagen arthritis in rats. Clinical and experimental rheumatology 1990;**8**(2):167-70
39. Zhang L, Li P, Song S, et al. Comparative efficacy of TACI-Ig with TNF-alpha inhibitor and methotrexate in DBA/1 mice with collagen-induced arthritis. Eur J Pharmacol 2013;**708**(1-3):113-23.
40. Saadat F, Cuzzocrea S, Di PR, et al. Effect of pyrimethamine in experimental rheumatoid arthritis. Med Sci Monit 2005;**11**(8):BR293-BR99 doi: 7042 [pii][published Online First: Epub Date]|.
41. O'Valle F, Peregrina M, Crespo-Lora V, et al. Osteoarticular Expression of Musashi-1 in an Experimental Model of Arthritis. Biomed Res Int 2015;**2015**:681456 doi: 10.1155/2015/681456 [doi][published Online First: Epub Date]|.
42. Joosten LA, Helsen MM, van de Loo FA, et al. Anticytokine treatment of established type II collagen-induced arthritis in DBA/1 mice. A comparative study using anti-TNF alpha, anti-IL-1 alpha/beta, and IL-1Ra. Arthritis and rheumatism 1996;**39**(5):797-809
43. Yang T, Wang Z, Wu F, et al. A variant of TNFR2-Fc fusion protein exhibits improved efficacy in treating experimental rheumatoid arthritis. PLoS computational biology 2010;**6**(2):e1000669.
44. Bendele A, McAbee T, Sennello G, et al. Efficacy of sustained blood levels of interleukin-1 receptor antagonist in animal models of arthritis: comparison of efficacy in animal models with human clinical data. Arthritis and rheumatism 1999;**42**(3):498-506.
45. Kim YH, Kang JS. Effect of methotrexate on collagen-induced arthritis assessed by micro-computed tomography and histopathological examination in female rats. Biomol Ther (Seoul) 2015;**23**(2):195-200.

46. Yao Y, Ding CZ, Fang Y. Combination of MTX and LEF attenuates inflammatory bone erosion by down-regulation of receptor activator of NF-kB ligand and interleukin-17 in type II collagen-induced arthritis rats. Rheumatol Int 2013;**33**(7):1845-53
47. Teramachi J, Kukita A, Li YJ, et al. Adenosine abolishes MTX-induced suppression of osteoclastogenesis and inflammatory bone destruction in adjuvant-induced arthritis. Lab Invest 2011;**91**(5):719-31.
48. Baggott JE, Morgan SL. Methotrexate and erythro-9-(2-hydroxynon-3-yl) adenine therapy for rat adjuvant arthritis and the effect of methotrexate on in vivo purine metabolism. Eur J Pharm Sci 2007;**31**(2):95-101 |.
49. Brauer R, Kette H, Henzgen S, et al. Influence of cyclosporin A on cytokine levels in synovial fluid and serum of rats with antigen-induced arthritis. Agents Actions 1994;**41**(1-2):96-98
50. Wooley PH, Whalen JD, Chapman DL, et al. The effect of an interleukin-1 receptor antagonist protein on type II collagen-induced arthritis and antigen-induced arthritis in mice. Arthritis and rheumatism 1993;**36**(9):1305-14
51. Brahn E, Peacock DJ, Banquerigo ML. Suppression of collagen-induced arthritis by combination cyclosporin A and methotrexate therapy. Arthritis and rheumatism 1991;**34**(10):1282-88
52. Quinn MA, Emery P. Window of opportunity in early rheumatoid arthritis: possibility of altering the disease process with early intervention. Clinical and experimental rheumatology 2003;**21**(5 Suppl 31):S154-7
53. van Nies JA, Krabben A, Schoones JW, et al. What is the evidence for the presence of a therapeutic window of opportunity in rheumatoid arthritis? A systematic literature review. Ann Rheum Dis 2014;**73**(5):861-70.
54. van Dongen H, van Aken J, Lard LR, et al. Efficacy of methotrexate treatment in patients with probable rheumatoid arthritis: a double-blind, randomized, placebo-controlled trial. Arthritis and rheumatism 2007;**56**(5):1424-32.
55. Verstappen SM, McCoy MJ, Roberts C, et al. Beneficial effects of a 3-week course of intramuscular glucocorticoid injections in patients with very early inflammatory polyarthritis: results of the STIVEA trial. Ann Rheum Dis 2010;**69**(3):503-9
56. Machold KP, Landewe R, Smolen JS, et al. The Stop Arthritis Very Early (SAVE) trial, an international multicentre, randomised, double-blind, placebo-controlled trial on glucocorticoids in very early arthritis. Ann Rheum Dis 2010;**69**(3):495-502.
57. Emery P, Durez P, Dougados M, et al. Impact of T-cell costimulation modulation in patients with undifferentiated inflammatory arthritis or very early rheumatoid arthritis: a clinical and imaging study of abatacept (the ADJUST trial). Ann Rheum Dis 2010;**69**(3):510-6.
58. Vossenaar ER, Nijenhuis S, Helsen MM, et al. Citrullination of synovial proteins in murine models of rheumatoid arthritis. Arthritis and rheumatism 2003;**48**(9):2489-500.

Chapter 8

Periodontal infection and induction of autoimmunity in rheumatoid arthritis: *Aggregatibacter actinomycetemcomitans*-induced hypercitrullination

Science Translational Medicine 2018 Mar 21;10(433).

Adapted from: Comment on "Aggregatibacter actinomycetemcomitans–induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis"

Mikhail Volkov[1], Jacqueline Dekkers[1], Bruno G. Loos[2], Sergio Bizzarro[2], Thomas W.J. Huizinga[1], Helle A. Praetorius[3], René E.M. Toes[1], Diane van der Woude[1]

1. Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands
2. Department of Periodontology, Academic Centre for Dentistry Amsterdam, University of Amsterdam and Vrije Universiteit, Amsterdam, the Netherlands
3. Department of Biomedicine, Aarhus University, Aarhus, Denmark

Periodontal disease is a chronic inflammatory condition affecting the tissues surrounding the teeth and is caused by dysbiosis of the oral microbiota. Periodontal disease and RA are closely linked as both diseases are characterized by localized chronic inflammatory reactions. Clinical studies have shown that periodontitis is more prevalent in patients witch active RA than in health individuals; conversely, the prevalence of RA is also higher in individuals with periodontitis than in those without periodontitis. (1)

The periodontal pathogen *Aggregatibacter actinomycetemcomitans* (Aa) seems to represent a link between periodontal infection and autoimmunity. Recently, the group of Konig et al. describe a potential explanation for the link between periodontal infection and rheumatoid arthritis (RA) (2). They identify a specific periodontitis-associated bacterium: *Aggregatibacter actinomycetemcomitans* (Aa), which via its lytic toxin (leukotoxin A: LtxA) can dysregulate the activity of citrullinating enzymes in neutrophils. The thus-generated citrullinated autoantigens are the target of a key autoantibody system in RA: anti-citrullinated protein antibodies (ACPA). Furthermore, the authors report that the effect of the most important genetic risk factor for RA: the human leukocyte antigen-DRB1 shared epitope (HLA SE) alleles, was limited to RA patients who had been exposed to Aa as determined by seropositivity to LtxA in a set of 194 RA patients. Based on these findings, the authors hypothesize that LtxA may be a key factor in the initiation of the RA-specific anti-citrullinated protein immune response in genetically predisposed individuals. In light of the crucial implications of this novel hypothesis for the current thinking regarding RA development, we aimed to replicate these findings.

To this end, we focused on two main questions: 1) is the increased exposure to Aa as measured by the presence of anti-LtxA-antibodies specific for RA, or also present in other forms of inflammatory arthritis? 2) can we replicate the finding that the association between HLA SE alleles and ACPA-positive RA is limited to the anti-LtxA-positive subset?

To answer these questions, we established an enzyme-linked immunosorbent assay (ELISA) against purified LtxA. The purification of LtxA was performed according to the method described by Reinholdt et al. (3), which is the same method carried out from the same clone of the strain of Aa (HK921; JP2 positive) in the same laboratory as in the manuscript by Konig et al. The activity of LtxA was confirmed in a haemolytic assay, to correspond to the normal range of freshly purified LtxA. To confirm that the presence of antibodies against LtxA was indeed a reflection of exposure to the bacterium Aa, we first tested serum samples from periodontitis-patients (free from any chronic diseases such as RA) whose subgingival lesions had been tested for the presence of Aa by culturing techniques (4). As depicted in Figure 1A, the levels of anti-LtxA-antibodies were clearly elevated in Aa-positive patients (median level (interquartile range (IQR): 8853 (5544; 14147)) versus Aa-negative patients (median level (IQR): 764 (523; 4636)) confirming the specificity of the ELISA (Mann-Whitney U-test p-value < 0.001).

Thus, we subsequently tested sera from 594 patients participating in the Leiden Early Arthritis Clinic with various different diagnoses, including RA according to the 1987 ACR criteria (5)**.** Furthermore, we also measured anti-LtxA-levels and positivity in a group of 156 healthy controls (without chronic illnesses) from the Leiden area. Figure 1B depicts the anti-LtxA-levels in these various groups and illustrates that anti-LtxA antibodies could be found in a substantial proportion of RA patients, but also in patients with other forms of arthritis. Serial dilutions of a mix of 3 strongly positive RA patients were used as a standard, and the lowest point of the linear part of the standard curve (2000 AU/ml) was defined as the cut-off. Next, we investigate whether within RA patients, anti-LtxA-antibodies were preferentially present within the ACPA-positive group, and whether there was an association with the HLA SE-alleles. As depicted in Figure 1C, neither of these associations could be found in our cohort. Furthermore, as can be seen in Table 1, the association between HLA DRB1 SE alleles and anti-CCP-positive RA was similar among RA patients positive and negative for anti-LtxA. Therefore, the effect of the HLA SE alleles appears not to be confined to the patient group positive for anti-LtxA-antibodies.

Table 1 The association of SE alleles with anti-CCP based on exposure to LtxA in patients with RA. Anti-LtxA, anti-leukotoxin antibodies as determined by ELISA; Anti-CCP, anti-cyclic citrullinated peptide antibody, cut-off for positivity >20 U; SE, HLA-DRB1 shared epitope allele

	Anti-LtxA-negative RA (n = 143)				**Anti-LtxA-positive (n = 189)**			
	SE-negative (n = 44)	**SE-positive (n = 99)**	**OR**	**P**	**SE-negative (n = 66)**	**SE-positive (n = 123)**	**OR**	**P**
Anti-CCP positivity, %	34	70	4,45	0.0001	29	68	5,33	<0.0001

In summary, in this large cohort of arthritis patients, anti-LtxA-antibodies were not specifically associated with RA, and within RA patients, there was no association with the presence of ACPA or HLA SE alleles, in contrast to the findings of Konig et al. A possible explanation for these divergent results could be that the anti-LtxA-assay as used here, may have differed from the assay in the previous publication. However, the essential constituent (LtxA) and set-up of the assay was derived from the same source. Furthermore, we performed various controls e.g. in periodontitis patients, the results of which confirmed the validity of our assay.

It is possible that differences in proportions of positive patients reported in the original study by Konig et al and in our study could be due to differences in patient population between the United States and the Netherlands. It seems likely that differences in living environment, genetic background and referral strategy exist between these two countries. However, given the fact that Aa is a prevalent micro-organism causing periodontitis in both countries, it appears unlikely that population differences can explain the contrasting findings regarding the possible role of Aa as found by Konig versus us.

Although microbial influences may well be important in the development of RA, our results do not support a key role of exposure to LtxA originating from the periodontal pathogen Aa in linking the effect of the HLA SE alleles and periodontal disease to anti-citrullinated protein autoimmunity in RA.

Whilst the hypothesis of a causal link between periodontitis/periodontitis-associated microorganisms and RA seems appealing, it is also possible that RA patients are more frequently anti-LtxA-positive simply because they have more periodontitis as is known from epidemiological studies. We hope our findings can contribute to the discussion about the origins of autoimmunity in RA, and look forward to other replication experiments regarding these intriguing observations.

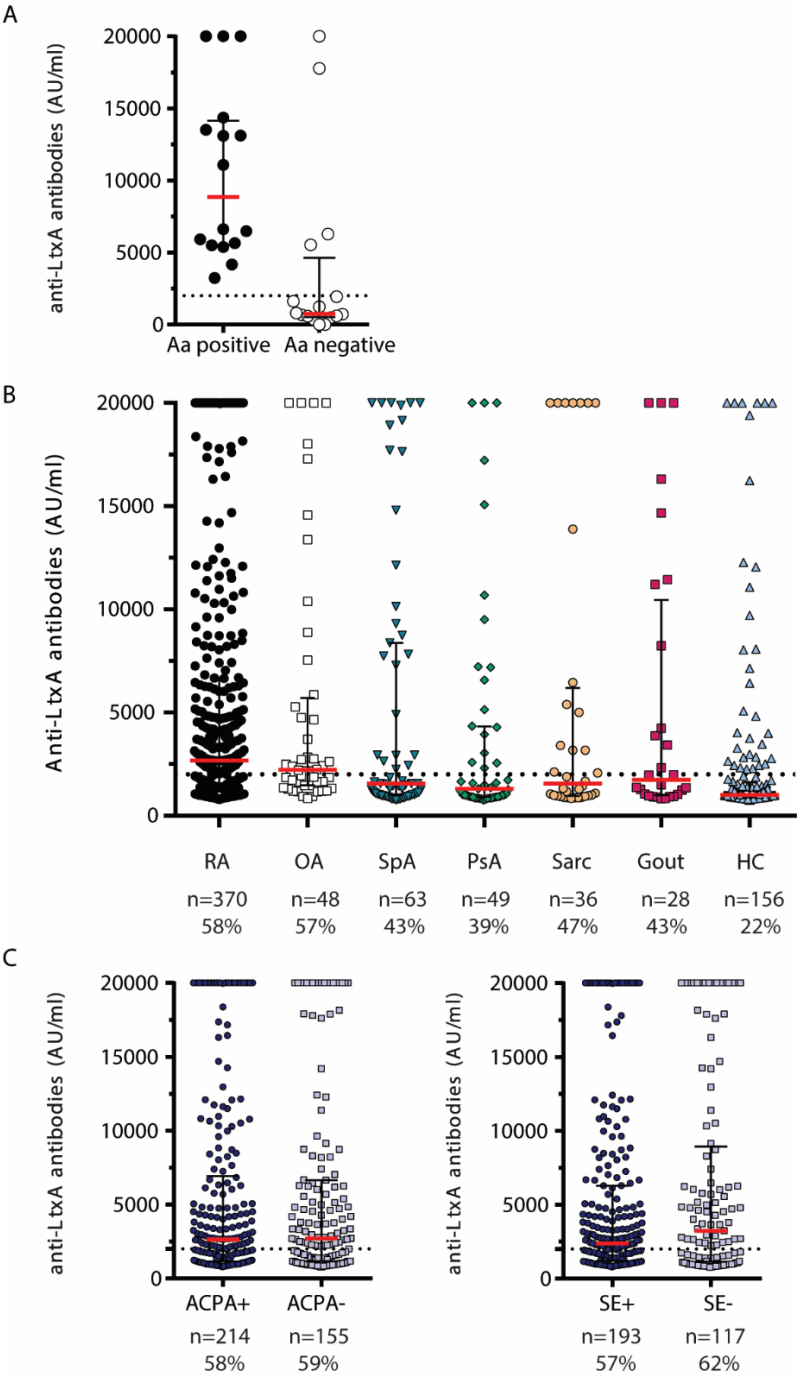

A
anti-LtxA antibodies (AU/ml)
20000
15000
10000
5000
0
Aa positive
Aa negative
B
Anti-LtxA antibodies (AU/ml)
20000
15000
10000
5000
0
RA
OA
SpA
PsA
Sarc
Gout
HC
n=370
58%
n=48
57%
n=63
43%
n=49
39%
n=36
47%
n=28
43%
n=156
22%
C
anti-LtxA antibodies (AU/ml)
20000
15000
10000
5000
0
ACPA+
ACPA-
n=214
58%
n=155
59%
anti-LtxA antibodies (AU/ml)
20000
15000
10000
5000
0
SE+
SE-
n=193
57%
n=117
62%

Figure 1. Serum antibodies to leukotoxin A. (A) Serum antibodies to LtxA were measured in periodontitis patients with and without Aa infection (n=16 per group) confirmed by culturing. (B) Distribution of anti-LtxA antibodies in sera of 594 patients suffering from early arthritis and 156 controls. Levels of anti-LtxA antibodies in the serum of each individual are shown. (C) Serum antibodies to LtxA were measured in RA patients stratified for the presence of anti-CCP2 antibodies, ACPA positive (n=214) and ACPA negative (n=155) RA patients (left panel). Presence of anti-LtxA antibodies in sera of RA patients carrying HLA-DRB1 shared epitope alleles, SE positive (n=243) and SE negative (n=117) RA patients (right panel).
LtxA Leukotxin A, RA Rheumatoid arthritis, Aa *Aggregatibacter actinomycetemcomitans*, OA Inflammatory osteoarthritis, SpA Spondylarthritis with peripheral arthritis, PsA Psoriatic arthritis, Sarc Sarcoidosis, ACPA anti-citrullinated protein antibodies, SE shared epitope. Red lines indicate the median level per group. The dashed line indicates the cut-off. The number of patients per group and percentage of patients positive according to the cut-off are shown underneath.

References

1. Potempa J, Mydel P, Koziel J**.** The case for periodontitis in the pathogenesis of rheumatoid arthritis. Nat Rev Rheumatol. 2017;13(10):606-20.
2. Konig MF, Abusleme L, Reinholdt J, Palmer RJ, Teles RP, Sampson K, et al. Aggregatibacter actinomycetemcomitans-induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis. Science Translational Medicine. 2016;8(369).
3. Reinholdt J, Poulsen K, Brinkmann CR, Hoffmann SV, Stapulionis R, Enghild JJ, et al. Monodisperse and LPS-free Aggregatibacter actinomycetemcomitans leukotoxin: Interactions with human beta(2) integrins and erythrocytes. Biochimica Et Biophysica Acta-Proteins and Proteomics. 2013;1834(2):546-58.
4. Bizzarro S, Van der Velden U, Loos BG**.** Local disinfection with sodium hypochlorite as adjunct to basic periodontal therapy: a randomized controlled trial. Journal of Clinical Periodontology. 2016;43(9):778-88.
5. de Rooy DP, van der Linden MP, Knevel R, Huizinga TW, van der Helm-van Mil AH**.** Predicting arthritis outcomes--what can be learned from the Leiden Early Arthritis Clinic? Rheumatology.(Oxford). 2011;50(1):93-100.

Chapter 9

Discussion and general summary

Autoantibodies against post-translationally modified (PTM) proteins are a hallmark of RA. The reason why an immune response starts against PTM proteins is not known. However, it appears crucial to obtain understanding on the breach of tolerance towards PTM proteins as the immune response against these proteins has been intimately implicated in disease-pathogenesis. In this thesis we report that exposure to modified self- and foreign proteins can lead to the generation of anti-modified protein antibodies (AMPA) which are directed against PTMs. Exposure of mice to carbamylated self- and foreign antigens can lead to the formation of self-reactive anti-CarP antibodies (chapter 3) (1). We also found that exposure to carbamylated self-proteins is sufficient to trigger primary immune responses, including autoantibody formation, T cell activation and cytokine production (chapter 4) (2). The studies in chapter 5 describe the observation that vaccinating mice with an acetylated protein leads to the formation of auto-antibodies against carbamylated proteins (anti-CarP antibodies) as well, indicating that different AMPA-responses can evolve from exposure to only one type of modified protein (3). In addition, we found that AMPA from RA-patients purified against one PTM can recognize different classes of PTMs. Chapter 6 describes the relationship between autoantibody status and treatment response. We found that in newly diagnosed RA patients who are receiving methotrexate, autoantibody status was not associated with the chance of achieving early remission. This indicates that methotrexate is effective as initial treatment strategy regardless of autoantibody status (4). The studies described in chapter 7 show that both prophylactic and pre-arthritis treatment strategies lead to a significant reduction of arthritis severity in animal models (5). Chapter 8 describes studies regarding the link between periodontal infection and autoimmunity. In a large cohort of arthritis patients we found that anti-LtxA antibodies were not specifically associated with RA. In addition, there was no association between ACPA or HLA SE alleles among RA patients. Together, these studies highlight that the different anti-modified protein antibody responses present in RA could have a common origin which could be potentially implicated in disease pathogenesis. Regarding the described studies, several issues are worthwhile to be discussed in more detail.

Detection of AMPA responses: specificity and reproducibility

For the detection of AMPA we used anti-carbamylated protein, anti-citrulline and anti-acetylated lysine ELISA antibody techniques (1-3). As these ELISA techniques are predominately used for the identification of these autoantibodies, its accuracy and reproducibility are crucial for the validity of our conclusions. The antigens used for identification are fetal calf serum, serum albumin and fibrinogen. These antigens were chosen as they are readily available, relatively cheap and easily to modify (i.e. carbamylate, citrullinate or acetylate) in a consistent manner. The non-modified counter parts served as

control. After protein modification by carbamylation, citrullination or acetylation, the presence of the modification on the antigens was confirmed by mass-spectrometry. This is a crucial step in order to confirm the solely presence of one particular modification in the absence of other, structurally similar, PTMs. Mass-spectrometry is a very specific method for compound identification as the citrulline and homocitrulline are distinguished based on their position within the protein (they are derived from either an arginine or a citrulline). Furthermore, homocitrulline differs from citrulline by 14 Da. Although mass-spectrometry is a specific technique, it is not a quantitative method. Therefore, we used commercial antibodies recognizing citrullinated, carbamylated or acetylated proteins to quantify the extent of modification present.
We optimized in-house ELISAs to determine the presence of protein modifications and by titrating these antibodies the extent of modifications present can be estimated (although not in absolute terms). To confirm the reproducibility of our assays we performed several internal control experiments. Our previous studies with human sera showed an intra- and inter-assay variability of anti-carbamylated fetal calf serum (anti-Ca-FSC) and anti-carbamylated fibrinogen (anti-Ca-FCS) of around 10-15% (6). Using sera of immunized mice, the intra- and inter-assay variability was more consistent (around 5-10%). In repeated measurements the chance of a false-positive sample in non-immunised mice was generally around 2-5%. It is important to acknowledge that there remains a certain degree of intra assay variation which can be considered as random variations due to the methodology of our assays (e.g. proteins rather than structurally defined peptides).

AMPA responses and cross-reactivity

Since homocitrulline structurally highly resembles citrulline, it is possible that anti-CarP antibodies are cross-reactive to citrullinated antigens. It has been reported that ACPA can bind peptides or proteins containing homocitrulline (7-9), however this does not directly implicate that antibodies in general cannot be specific for these PTMs. Previously, we have shown that human polyclonal antibodies can be specific for either carbamylation or citrullination (10). Additional studies indicate that the specificity towards citrullinated or homocitrulline does exist, but is dependent on the sera used (11). To test for cross-reactivity of polyclonal anti-CarP antibodies with carbamylated proteins, we performed inhibition assays with the same protein used both as antigen and inhibitor (1). This resembles a relevant positive control to show that at the chosen inhibitor concentration, inhibition is indeed present. The relevance of the inhibition assays lies in the comparison to the other modified proteins. Our data show that other, structurally unrelated, modified proteins are also able to inhibit the anti-CarP-response generated.

The studies in chapter 5 describe the exciting observation that vaccinating mice with e.g. an acetylated protein leads to the formation of anti-CarP antibodies as well (3), indicating that different AMPA-responses can evolve from exposure to only one type of modified protein. In our studies we found that the avidity of the antibody response to carbamylated fibrinogen and acetylated fibrinogen was similar after immunization with, respectively, carbamylated ovalbumin (RAI 64.3%) or acetylated ovalbumin (RAI 43.1%) (3). We subsequently found that avidity of the AMPA-response is highest towards the respective modified antigen used for immunization. Discrepancies in the degree of cross-reactivity may be a result of proteins that contain different numbers and locations of PTMs. So far a well-defined chemical construct recognized by anti-CarP antibodies which could be used as a pan inhibitor for inhibition experiments has not been identified so far.

In additional experiments we studied the polyclonal responses present in human serum. We used a biotinylated CCP2 peptide bound column to isolate anti-CCP2 antibodies and biotinylated Ca-FCS for the isolation anti-CarP antibodies in patients serum samples. After purification, ACPA were strongly enriched for reactivity towards both carbamylated and acetylated vimentin peptides (3). Following our studies on the cross-reactivity of purified ACPA from RA patients, our team has studied whether monoclonal ACPA (12) can cross-react to acetylated antigens and other PTM proteins. Interestingly, we recently observed that this human monoclonal ACPA is able to react not only to citrullinated, but also to acetylated fibrinogen (data in preparation). Carbamylated fibrinogen was not recognized. Together these data are important as they show for the first time that ACPA can be cross-reactive towards acetylated proteins as well. The relative cross-reactivity between ACPA, anti-CarP and anti-acetylated lysine antibodies suggests that these autoantibodies may originate from the same B-cell population. Acetylated lysine does not resemble citrulline but bears similarity to homocitrulline except at the side chain terminal amine, which is replaced by a methyl moiety.

In our studies we observed that ACPA cannot only cross-react to some extent to carbamylated proteins, but also to acetylated proteins. In line with our data, others have shown that reactivity to citrullinated vimentin peptides was blocked by preincubation with the soluble citrullinated peptide as expected, with weak inhibition of binding by carbamylated (23%) and acetylated (17%) vimentin peptides. Similar specificity and low cross-reactivity was observed for the anti-acetylated vimentin antibodies as well as for anti-carbamylated vimentin where 32% of the binding was inhibited by preincubation with citrullinated soluble peptide (8). These results might be explained by the notion that AMPA consist of different auto-antibody families that are largely distinct, but that can also display a certain degree of cross-reactivity.

It is interesting to note that a defined antigen-receptor can recognize both citrullinated and acetylated antigens despite the structural dissimilarities of these two antigens. This indicates that different AMPA-responses can evolve from exposure to only one type of

modified protein. These data could represent a paradigm shift explaining the induction of AMPA-responses in RA since they show that the inciting antigen responsible for the induction of e.g. anti-CarP-antibodies does not have to be citrullinated or carbamylated, but could be represented by an acetylated protein. Increasing evidence suggests that mucosal surfaces, specifically the periodontium, the gut and the lungs, as sites of disease initiation of RA and indicate the microbiome as an important driver of the initiation of auto-immunity. In this respect, especially protein–acetylation by bacteria might now also be incriminated in the induction of auto-antibody responses against PTM proteins.
In our future studies it would be interesting to generate and test more monoclonal ACPA for the antigen recognition profile. In addition, it would be of interest to establish a method to purify anti-acetylated protein antibodies to determine if- and to what extent human polyclonal AMPAs are cross-reactive towards different modified antigens. Together with current data, these future experiments will provide valuable insight into the magnitude and extent of the cross-reactive nature of human AMPAs towards three different modified antigens.

AMPA responses in mice

In chapter 4 we showed that stimulation of spleen cells with dendritic cells pulsed with carbamylated mouse albumin led to the induction of a strong T cell response, cytokine production and proliferation. Stimulation of spleen cells with homocitrulline containing peptides identified by mass spectrometry resulted in a PTM specific T-cell response. These findings suggest that posttranslational modification of self-proteins can result in 'new' antigens for which immune tolerance does not exist. It was recently shown by others that, sera of mice exposed to tobacco smoke contains anti-CarP antibodies and increased amounts of carbamylated vimentin (13). Although the formation of PTMs seems to promote tolerance loss and autoimmunity in RA, it is still unknown whether AMPA responses are directly pathogenic or a marker for inflammation.
ACPA are considered to be a highly important serological marker in RA patients, however, their role and importance in mouse models of arthritis remains far less clear. In mouse models of RA the antibody dependent collagen-induced arthritis (CIA) and K/BxN model clearly require B cells, and the sera can be used to transfer disease, confirming a direct pathogenic role of humoral immunity in arthritis (14, 15). In the CIA model, inflammatory arthritis is induced in genetically susceptible mice by immunization with type II collagen (CII) (16) which subsequently leads to the induction of autoreactive anti-CII antibodies (17, 18). There is conflicting evidence whether ACPA are involved in CIA (19). It has been reported that ACPA can be found in sera of mice with arthritis that did not receive additional vaccines with citrullinated antigens (20-22). However, data whether ACPA can induce or exacerbate

symptoms are limited as the majority of animal studies show that passive transfer of ACPAs alone does not induce arthritis (21, 23-25).
Our team has previously shown that anti-CarP antibodies, but not anti-CCP2 antibodies, are present in sera of DBA-1 mice and C57BL/6 with CIA. It was shown that the onset of arthritis was preceded by an increase of anti-CarP antibodies (26). These data are in line with the data that in asymptomatic blood bank donors there is a rise in anti-CarP levels prior to clinical onset of RA (27). For future studies it would be interesting to examine whether anti-acetylated lysine antibodies are also present in sera of mice with arthritis. We recently studied the role of anti-CarP antibodies in arthritis using our monoclonal anti-CarP antibody (data in preparation). In a pilot experiment we tested whether exposure to anti-CarP antibodies could exacerbate arthritis. DBA-1 mice were injected with CFA and CII according to protocol and later received additional anti-CarP antibodies. In these first experiments, we observed no exacerbation of symptoms upon transfer of anti-CarP antibodies. It would be interesting in the future to study the role of AMPA in arthritis using additional monoclonal antibodies such as anti-acetylated lysine antibodies.
In summary, the hypothesis that AMPA responses play a key role in the pathogenesis of RA needs solid and repeatable animal data. The identification of additional PTM responses besides ACPA, such as anti-carbamylate and acetylated protein antibodies, enables us to study the induction and role of these AMPA in animal models of arthritis.

Autoantibodies in the pathogenesis of RA

Despite many years of intensive research, the pathogenesis of RA remains elusive. It appears that breach of tolerance towards PTM proteins and the generation of AMPA is intimately implicated in disease-pathogenesis. Genetic predisposition and environmental factors such as smoking are thought to contribute to this break of tolerance. However, asymptomatic individuals can harbour autoantibodies without developing RA for many years, which suggests that the sole presence of an autoantibody is insufficient to trigger the onset of RA (27).
Previous studies have shown that ACPA-producing B-cells are found to be enriched in synovial fluid (28, 29), suggesting that ACPA can be produced locally and directly contribute to synovial inflammation. So far, little information is currently available on the nature of carbamylate proteins that are present in the (inflamed) joint. Using mass-spectrometry we have been able to identify carbamylated serum albumin (1) and alfa-1-anti trypsin (30) in the synovial compartment of RA patients. Recently, other carbamylated proteins have been identified in patients with renal disease (31, 32), indicating that it is likely that also other carbamylated proteins can be carbamylated in RA patients. It will be interesting to see whether the nature and location of carbamylated proteins in the synovial compartment is similar or distinct from the citrullinated proteins found in the joint. Studies in chapter 6

describe the relationship between autoantibody status and remission in newly diagnosed RA-patients treated with first-line methotrexate. Methotrexate is the most widely used anti-rheumatic drug in clinical practice (33). We found that in newly diagnosed RA patients who are receiving methotrexate, autoantibody status (ACPA and/or rheumatoid factor) was not associated with the chance of achieving early remission (4). In the future, patients may benefit from treatment tailored to “autoantibody status”.

It has been hypothesised that very early treatment initiation can prevent the development of RA, a so called “window-of -opportunity". Animal models provide a model to study the developing (auto)immune response at a very early disease phase. The translation of different stages of experimental arthritis to the evolution of human disease might provide valuable information regarding possibilities of disease prevention. Based on different studies in animal models we found that both prophylactic and pre-arthritis treatment strategies lead to a significant reduction of arthritis severity, chapter 7 (5). Autoantibody formation against type II collagen can be used as a marker to characterize different stages of disease. The time period in which auto-immunity is present and arthritis is still absent was referred to as the pre-arthritis period. Current animal research mainly focusses on testing anti-rheumatic drugs in established disease and preventive treatment strategies are not studied frequently. In an ideal experiment, different interventions should be studied side-by-side in different disease phases and with a similar treatment schedule to be able to compare efficacy.

Protein modification and periodontal disease appear to be relevant for the pathogenesis of RA, however, how these processes may be mechanistically related remains poorly defined. The periodontal pathogen Aggregatibacter actinomycetemcomitans (Aa) seems to represent a link between periodontal infection and autoimmunity. It was previously shown by others that Aa is capable to induce neutrophil hypercitrullinations through the secretion of leukotoxin A (LtxA), a bacterial pore-forming toxin that induces calcium influx and subsequent hyperactivation of PAD enzymes in the neutrophil. The studies in chapter 8 describe that in a large cohort of arthritis patients, anti-LtxA antibodies (used as surrogate marker of Aa infection) were not specifically associated with RA but could also be identified in other forms of inflammatory arthritis (34). In addition, we found no association between ACPA or HLA SE alleles in contrast to the previous study (33). Differences in cohorts and methodology may account for the difference in effect size. In our cohort the anti-CCP positivity is 58% (Leiden) versus 77% (Baltimore). The Leiden Early Arthritis Clinic includes patients with recent onset arthritis in whom definitive diagnosis are established after1 year of follow up. In contrast, the Baltimore cohort includes a wide range of disease durations, form early disease to decades. Regarding methodology, the cut-off for positivity was based on the lowest point of the linear part of the standard curve (2000 arbitrary units/ml, 75% specificity). It remains important to emphasize that despite not affecting comparisons of

median antibody concentrations between groups, a degree of uncertainty in cut-off selection can alter any interaction analyses.
Many bacterial species are able to acetylate proteins (35), including bacteria proposed as link between periodontal infection and RA (36). Disturbances of the microbiome, for example during infection, could lead to the increased formation of acetylated proteins which are detected by the immune system and thereby contribute to the induction of AMPA-responses. Microbe-specific T cells could initially help the B cells recognize the microbe-derived modified protein and thereby contributing to isotype-switching and somatic hypermutation.

Final conclusions

In conclusion, we showed that exposure to modified self- and foreign proteins can lead to the generation of AMPA. The observation that exposure to an acetylated protein leads to the formation of auto-antibodies against carbamylated proteins as well, indicates that different AMPA-responses can evolve from exposure to only one type of modified protein. Understanding the full AMPA response, the triggers that drive AMPA production, their mutual cross-talk and the pathways by which AMPA and/or AMPA expressing B cells possibly contribute to RA might – in the long run- allow interventions that prevent disease development, a highly desirable goal in the quest against rheumatic diseases.

References

1. Dekkers JS, Verheul MK, Stoop JN, Liu B, Ioan-Facsinay A, van Veelen PA, et al. Breach of autoreactive B cell tolerance by post-translationally modified proteins. Ann Rheum Dis. 2017;76(8):1449-57.
2. J.S. Dekkers JNS, L.A. Trouw, R.E.M. Toes**.** Carbamylation of self-antigens facilitates a breach in T-cell tolerance
3. J.S. Dekkers¥ ASBK, , M. Volkov, A.M. Dorjee, L. Hafkenscheid, A. Kempers, M.A.M. van Delft, G.M.C. Janssen, P.A. van Veelen, T.M.W. Huizinga, D. van der Woude, R.E.M. Toes**.** Different classes of Anti-Modified Protein Antibodies are induced upon exposure to antigens expressing one type of modification.
4. Dekkers JS, Bergstra SA, Chopra A, Tikly M, Fonseca JE, Salomon-Escoto K, et al. Autoantibody status is not associated with early treatment response to first-line methotrexate in patients with early rheumatoid arthritis. Rheumatology (Oxford). 2018.
5. Dekkers JS, Schoones JW, Huizinga TW, Toes RE, van der Helm-van Mil AH**.** Possibilities for preventive treatment in rheumatoid arthritis? Lessons from experimental animal models of arthritis: a systematic literature review and meta-analysis. Ann Rheum Dis. 2017;76(2):458-67.
6. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci U S A. 2011;108(42):17372-7.
7. Scinocca M, Bell DA, Racape M, Joseph R, Shaw G, McCormick JK, et al. Antihomocitrullinated fibrinogen antibodies are specific to rheumatoid arthritis and frequently bind citrullinated proteins/peptides. J Rheumatol. 2014;41(2):270-9.
8. Juarez M, Bang H, Hammar F, Reimer U, Dyke B, Sahbudin I, et al. Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis. Ann Rheum Dis. 2016;75(6):1099-107.
9. Reed E, Jiang X, Kharlamova N, Ytterberg AJ, Catrina AI, Israelsson L, et al. Antibodies to carbamylated alpha-enolase epitopes in rheumatoid arthritis also bind citrullinated epitopes and are largely indistinct from anti-citrullinated protein antibodies. Arthritis Res Ther. 2016;18(1):96.
10. Shi J, Willemze A, Janssen GM, van Veelen PA, Drijfhout JW, Cerami A, et al. Recognition of citrullinated and carbamylated proteins by human antibodies: specificity, cross-reactivity and the 'AMC-Senshu' method. Ann Rheum Dis. 2013;72(1):148-50.
11. Verheul MK, van Veelen PA, van Delft MAM, de Ru A, Janssen GMC, Rispens T, et al. Pitfalls in the detection of citrullination and carbamylation. Autoimmun Rev. 2018;17(2):136-41.
12. van de Stadt LA, van Schouwenburg PA, Bryde S, Kruithof S, van Schaardenburg D, Hamann D, et al. Monoclonal anti-citrullinated protein antibodies selected on citrullinated fibrinogen have distinct targets with different cross-reactivity patterns. Rheumatology. 2013;52(4):631-5.

13. Ospelt C, Bang H, Feist E, Camici G, Keller S, Detert J, et al. Carbamylation of vimentin is inducible by smoking and represents an independent autoantigen in rheumatoid arthritis. Ann Rheum Dis. 2017;76(7):1176-83.
14. Christensen AD, Haase C, Cook AD, Hamilton JA**.** K/BxN Serum-Transfer Arthritis as a Model for Human Inflammatory Arthritis. Front Immunol. 2016;7:213.
15. Nandakumar KS, Holmdahl R**.** Collagen antibody induced arthritis. Methods Mol Med. 2007;136:215-23.
16. Brand DD, Kang AH, Rosloniec EF**.** Immunopathogenesis of collagen arthritis. Springer Semin Immunopathol. 2003;25(1):3-18.
17. Holmdahl R, Andersson M, Goldschmidt TJ, Gustafsson K, Jansson L, Mo JA**.** Type II collagen autoimmunity in animals and provocations leading to arthritis. Immunol Rev. 1990;118:193-232.
18. Pearson CM**.** Development of arthritis, periarthritis and periostitis in rats given adjuvants. Proc Soc Exp Biol Med. 1956;91(1):95-101.
19. Vossenaar ER, Nijenhuis S, Helsen MM, van der Heijden A, Senshu T, van den Berg WB, et al. Citrullination of synovial proteins in murine models of rheumatoid arthritis. Arthritis Rheum. 2003;48(9):2489-500.
20. Kidd BA, Ho PP, Sharpe O, Zhao X, Tomooka BH, Kanter JL, et al. Epitope spreading to citrullinated antigens in mouse models of autoimmune arthritis and demyelination. Arthritis Res Ther. 2008;10(5):R119.
21. Kuhn KA, Kulik L, Tomooka B, Braschler KJ, Arend WP, Robinson WH, et al. Antibodies against citrullinated proteins enhance tissue injury in experimental autoimmune arthritis. J Clin Invest. 2006;116(4):961-73.
22. Forster M, Raposo B, Ekman D, Klaczkowska D, Popovic M, Nandakumar KS, et al. Genetic control of antibody production during collagen-induced arthritis development in heterogeneous stock mice. Arthritis Rheum. 2012;64(11):3594-603.
23. Harre U, Georgess D, Bang H, Bozec A, Axmann R, Ossipova E, et al. Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. J Clin Invest. 2012;122(5):1791-802.
24. Krishnamurthy A, Joshua V, Haj Hensvold A, Jin T, Sun M, Vivar N, et al. Identification of a novel chemokine-dependent molecular mechanism underlying rheumatoid arthritis-associated autoantibody-mediated bone loss. Ann Rheum Dis. 2016;75(4):721-9.
25. Wigerblad G, Bas DB, Fernades-Cerqueira C, Krishnamurthy A, Nandakumar KS, Rogoz K, et al. Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism. Ann Rheum Dis. 2016;75(4):730-8.
26. Stoop JN, Liu BS, Shi J, Jansen DT, Hegen M, Huizinga TW, et al. Antibodies specific for carbamylated proteins precede the onset of clinical symptoms in mice with collagen induced arthritis. PLoS One. 2014;9(7):e102163.
27. Shi J, van de Stadt LA, Levarht EW, Huizinga TW, Hamann D, van Schaardenburg D, et al. Anti-carbamylated protein (anti-CarP) antibodies precede the onset of rheumatoid arthritis. Ann Rheum Dis. 2014;73(4):780-3.

28. Snir O, Widhe M, Hermansson M, von Spee C, Lindberg J, Hensen S, et al. Antibodies to several citrullinated antigens are enriched in the joints of rheumatoid arthritis patients. Arthritis Rheum. 2010;62(1):44-52.
29. Amara K, Steen J, Murray F, Morbach H, Fernandez-Rodriguez BM, Joshua V, et al. Monoclonal IgG antibodies generated from joint-derived B cells of RA patients have a strong bias toward citrullinated autoantigen recognition. J Exp Med. 2013;210(3):445-55.
30. Verheul MK, Yee A, Seaman A, Janssen GM, van Veelen PA, Drijfhout JW, et al. Identification of carbamylated alpha 1 anti-trypsin (A1AT) as an antigenic target of anti-CarP antibodies in patients with rheumatoid arthritis. J Autoimmun. 2017;80:77-84.
31. Berg AH, Drechsler C, Wenger J, Buccafusca R, Hod T, Kalim S, et al. Carbamylation of serum albumin as a risk factor for mortality in patients with kidney failure. Sci Transl Med. 2013;5(175):175ra29.
32. Desmons A, Jaisson S, Pietrement C, Rieu P, Wynckel A, Gillery P**.** Homocitrulline: a new marker for differentiating acute from chronic renal failure. Clin Chem Lab Med. 2016;54(1):73-9.
33. Smolen JS, Landewe R, Breedveld FC, Dougados M, Emery P, Gaujoux-Viala C, et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs. Ann Rheum Dis. 2010;69(6):964-75.
34. Volkov M, Dekkers J, Loos BG, Bizzarro S, Huizinga TWJ, Praetorius HA, et al. Comment on "Aggregatibacter actinomycetemcomitans-induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis". Sci Transl Med. 2018;10(433).
35. Ouidir T, Kentache T, Hardouin J**.** Protein lysine acetylation in bacteria: Current state of the art. Proteomics. 2016;16(2):301-9.
36. Butler CA, Veith PD, Nieto MF, Dashper SG, Reynolds EC**.** Lysine acetylation is a common post-translational modification of key metabolic pathway enzymes of the anaerobe Porphyromonas gingivalis. J Proteomics. 2015;128:352-64.

Chapter 10

Nederlandse Samenvatting

Reumatoïde artritis (RA) is een chronische, systemische, auto-immuunziekte die wordt gekenmerkt door een chronische ontsteking van de synoviale gewrichten en de aanwezigheid van auto-antistoffen in het bloed. Belangrijk voor de klinische praktijk zijn anti-gecitrullineerde eiwit antilichamen (ACPA) en reuma factor (RF). Deze twee antilichamen zijn een onderdeel van de 2010 American College of Rheumatology / European League Against Rheumatism classificatiecriteria voor RA. ACPA binden aan eiwitten die een post-translationele modificatie hebben ondergaan. Citrullinatie is een chemische verandering waardoor het aminozuur arginine wordt omgezet in citrulline. Deze chemische verandering wordt gekatalyseerd door de zogenaamde peptidylarginine-de-iminase (PAD) enzymen. RF herkent het Fc gedeelte van IgG, een onderdeel betrokken bij complement activatie. Zowel ACPA als RF zijn diagnostische markers voor RA en de aanwezigheid van deze markers is voorspellend voor het ontwikkelen van RA. ACPA en RF kunnen lange tijd aanwezig zijn in het bloed van patiënten, zelfs jaren voordat symptomen ontstaan en RA zich klinisch manifesteert. De aanwezigheid van ACPA verhoogt bij patiënten met gewrichtsklachten de kans op de ontwikkeling van RA aanzienlijk, terwijl bij gediagnosticeerde RA patiënten de aanwezigheid van deze auto-antistoffen een meer agressief karakter van de ziekte voorspelt.

Naast ACPA zijn er recent ook andere RA-specifieke auto-antistoffen ontdekt die gericht zijn tegen post-translationeel gemodificeerde eiwitten. Deze antistoffen worden collectief beschreven als anti-gemodificeerde eiwit antistoffen (AMPA). AMPA kunnen naast citrulline ook gericht zijn tegen andere eiwit modificaties als homocitrulline en acetyllysine. De aanwezigheid van verschillende AMPA in RA suggereert een gezamenlijke biologische oorsprong. Net als citrullinatie zijn carbamylatie en acetyllatie post-translationele modificaties die onder normale omstandigheden voorkomen. Desondanks vormt slechts in een deel van de mensen, en met name RA patiënten, auto-antistoffen tegen eiwitten met deze modificaties. Het is dus van essentieel belang om te begrijpen waarom mensen antistoffen ontwikkelen die gericht zijn tegen dergelijke eiwitmodificaties die onder normale condities ook plaatsvinden.

Carbamylatie is een posttranslationele modificatie waarbij lysines worden gemodificeerd in homocitrullines onder invloed van cyanaat. De moleculaire structuur van homocitrulline lijkt veel op die van citrulline en is slechts één methyleen groep langer dan citrulline. Antistoffen gericht tegen gecarbamyleerde eiwitten worden beschreven als anti-gecarbamyleerde eiwit antilichamen (anti-CarP). Anti-CarP antistoffen komen voor bij circa 45% van de patiënten met een vroege vorm van RA. Daarnaast kunnen deze antistoffen ook gevonden worden bij proefdiermodellen met artritis. Kort geleden zijn antistoffen tegen acetyllysine geïdentificeerd als RA-specifieke AMPA. Deze antistoffen zijn aantoonbaar in het bloed bij 40% van de RA patiënten, met name in de ACPA positieve groep.

Het doel van dit proefschrift was om de onderliggende immuunregulatie en doorbreking van tolerantie tegen gemodificeerde eiwitten in RA te ontrafelen. AMPA zijn kenmerkend voor RA en worden verondersteld een rol te spelen in de onderliggende pathogenese.

In **hoofdstuk 2** beschrijven we gebaseerd op verschillende studies dat auto-immuniteit tegen gecitrullineerde eiwitten al reeds in een preklinische fase van RA aanwezig is. Daarnaast laten de studies zien dat de ACPA respons zich verder ontwikkelt over tijd en dat adequate behandeling van artritis kan leiden tot een andere samenstelling van ACPA of lagere titers. Auto-immuniteit kan ontstaan als gevolg van falen of doorbreken van mechanismen die aan de basis liggen van zelf tolerantie van B-cellen, T-cellen of beide.

Hoofdstuk 3 beschrijft het onderzoek naar het doorbreken van B-cel tolerantie. Blootstelling van proefdieren aan gecarbamyleerde eiwitten leidde tot de productie van anti-CarP antistoffen in het bloed. Daarnaast vonden we dat deze geïnduceerde anti-CarP antistoffen kruisreactief waren tegen andere gecarbamyleerde eiwitten, waarmee we auto-immuniteit aantoonden. Deze observatie laat zien dat het doorbreken van zelf tolerantie van B cellen door gemodificeerde lichaamsvreemde antigenen kan leiden tot kruisreactieve antilichamen tegen gemodificeerde lichaamseigen eiwitten. Voor de volledige activatie en differentiatie van rijpe B cellen zijn twee signalen nodig (1) interactie van oppervlakte-immuunglobuline op de B cel met het antigen en (2) co-stimulatie door middel van CD40L-CD40-interactie en cytokine productie door antigeen specifieke CD4+ T helper cellen. In **hoofdstuk 4** hebben we verder onderzoek gedaan naar het doorbreken van T cel tolerantie voor gecarbamyleerde auto-antigenen. Muizen werden gevaccineerd met gecarbamyleerd muizen albumine en niet-gemodificeerd albumine. Na vaccinatie met gecarbamyleerd albumine konden wij antigeen specifieke T cellen aantonen gericht tegen carbamyleerd albumine. Deze observatie ondersteunt de hypothese dat post-translationele modificaties kunnen fungeren als een “hapteen” (een klein molecuul wat kan dienen als epitoop en niet zelf in staat is om een antistofrespons op te wekken) en op deze manier een bijdrage leveren aan het ontstaan van AMPA.

Hoofdstuk 5 beschrijft het onderzoek naar de basis van AMPA reacties en AMPA producerende B cellen. Om antistoffen te kunnen detecteren gericht tegen geacetyleerde eiwitten hebben we een ELISA opzet gebaseerd op geacetyleerd ovalbumine en fibrinogeen. In deze studies onderzochten wij of blootstelling aan één soort post-translationele modificatie kan leiden tot de ontwikkeling van antistoffen gericht tegen andere modificaties. Na vaccinatie met gecarbamyleerde eiwitten konden wij in het bloed niet alleen anti-CarP antistoffen aantonen maar ook antistoffen gericht tegen geacetyleerde eiwitten. Vervolgens immuniseerden wij muizen met geacetyleerde eiwitten. Naast AAPA konden wij ook antilichamen aantonen gericht tegen andere PTM. Vervolgens vroegen wij ons af of gezuiverd ACPA naast citrulline reactiviteit ook reactief is tegen geacetyleerde en gecarbamyleerde eiwitten. Door antistoffen in het bloed van RA patiënten gezuiverd voor citrulline reactiviteit te isoleren en testen vonden wij dat deze antistoffen kruisreactief

kunnen zijn voor zowel acetyllysine als homocitrulline. Deze data geven aan dat in het serum van RA patiënten naast antilichamen die uniek reageren met ofwel gecitrullineerde eiwitten ofwel gecarbamyleerde eiwitten ook kruisreagerende populaties antistoffen aanwezig kunnen zijn. Vroege en agressieve interventie bij RA patiënten kan botschade voorkomen en vroege remissie induceren. De aanwezigheid van autoantistoffen kan een bruikbare biomarker zijn om patiënten te identificeren die van bepaalde behandelstrategieën wellicht meer baat hebben.

In de studies beschreven in **hoofdstuk 6** onderzochten we de associatie tussen auto-antistof status en het bereiken van vroege remissie bij recent gediagnosticeerde RA patiënten behandeld met methotrexaat. Voor ons onderzoek maakten wij gebruik van de internationale METEOR database wat bestaat uit data van verschillende reumatologische centra wereldwijd. Op basis van data van 1,826 RA patiënten concludeerden wij dat ongeacht de antistof status methotrexaat effectief is als inductie therapie.

Hoofdstuk 7 beschrijft het onderzoek gericht op de preklinische fase van de ziekte en de mogelijkheid voor preventieve behandelstrategieën voor RA. Recent onderzoek heeft aangetoond dat indien de behandeling van reumatoïde artritis binnen 12 weken na het ontstaan van klachten wordt gestart patiënten minder gewrichtsschade ontwikkelen en een grotere kans hebben op het bereiken van remissie. Hierop vormden wij de hypothese dat vroege interventie het ontstaan van chronische inflammatie en gewrichtsschade kan voorkomen en het ontstaan van ziekte kan voorkomen. Omdat de ziektefasen van artritis in proefdiermodellen duidelijk te karakteriseren zijn, besloten we om dit als model te gebruiken voor onze onderzoeksvraag. Door middel van een meta-analyse concludeerden wij dat zowel profylactische als preklinische behandelstrategieën kunnen leiden tot significante reductie van ziekte ernst.

De pathogenese van parodontitis en RA vertonen veel overeenkomsten, parodontitis wordt gekarakteriseerd door gingivitis en chronische inflammatie van het bot. Parodontitis kent een hogere prevalentie bij patiënten met RA en omgekeerd heeft RA een hogere prevalentie bij patiënten met parodontitis. Recent onderzoek toonde een verband aan tussen ACPA en de *Aggregatibacter actinomycetemcomitans* (Aa), een bacterie geassocieerd met parodontitis, en zijn lytische toxine (leutoxine A, Ltx-A) bij individuen met een genetische predispositie.

In **hoofdstuk 8** staan de studies beschreven naar de associatie tussen HLA-SE allelen en ACPA positieve RA wat eerder werd beschreven voor een anti-LxtA antistof positieve subset. Wij hebben hiervoor analyses uitgevoerd op 594 sera van patiënten uit het Leiden Early Arthritis Clinic (EAC) cohort, bestaande uit patiënten met alle vormen van vroege artritis zoals die in de polikliniek voorkomen. Op basis van deze sera konden wij concluderen dat de aanwezigheid van anti-LtxA antistoffen (als maat voor blootstelling aan Aa) niet specifiek is voor RA maar dat deze antistoffen ook voorkomen bij andere vormen van vroege artritis. Daarnaast vonden wij geen associatie tussen de aanwezigheid van ACPA of HLA SE allelen.

Ten slotte staat in **hoofdstuk 9** een samenvatting en discussie van alle onderzoeksresultaten beschreven en de mogelijkheden voor onderzoek in de toekomst.

Conclusies en toekomstperspectieven

De laatste jaren is er binnen het veld van RA een verschuiving opgetreden richting de vroege identificatie en vroege behandeling met als doel om de chroniciteit en zelfs de ontwikkeling van RA te voorkomen. Biologische markers als AMPA zijn niet alleen waardevol voor vroege identificatie maar kunnen inzicht leveren in de onderliggende immuun-regulatie en pathogenese. De beschreven onderzoeken in dit proefschrift geven inzicht in de rol van gemodificeerde eiwitten en doorbreking van tolerantie tegen gemodificeerde eiwitten, aspecten die kenmerkend zijn voor RA. In dit proefschrift staat beschreven dat auto-immuniteit tegen gecarbamyleerde eiwitten kan ontstaan als gevolg van het doorbreken van mechanismen die aan de basis liggen van zelf tolerantie zowel van B-cellen als T-cellen. Onze resultaten laten ook zien dat blootstelling aan andere gemodificeerde eiwitten kan leiden tot de productie van kruisreactieve antistoffen gericht tegen structureel andere modificaties. De methodologische aspecten in dit proefschrift hebben een fundament gelegd voor uitgebreidere studies naar de rol van post-translationele modificaties en het voorkomen van AMPA in patiëntengroepen. Toekomstige studies met monoklonale AMPA zouden meer inzicht kunnen geven in de mate van kruisreactiviteit en de mechanismen die aan basis van auto-immuniteit en tolerantiedoorbreking liggen. Het testen van monoklonale AMPA in proefdiermodellen van artritis kan inzicht geven in de rol van AMPA in het ontstaan van inflammatie en de ontwikkeling van artritis.
Samengevat, post-translationele modificaties en auto-antistoffen is een fascinerend en complex systeem. Toekomstige studies zullen gericht zijn om de “pathologische capaciteit” van de AMPA in kaart te brengen en de consequenties van de aanwezigheid van deze auto-antistoffen op het ziekteverloop van RA te achterhalen.

List of publications

Curriculum Vitae

Dankwoord

Edouard Manet (1832-1883), In the Conservatory (detail),
1879 Old National Gallery, Berlin, Germany

List of publications

Different classes of Anti-Modified Protein Antibodies are induced upon exposure to antigens expressing one type of modification.
J.S. Dekkers, A.S.B. Kampstra, M. Volkov, A.L. Dorjee, L. Hafkenscheid, A.C. Kempers, M. van Delft, T.Kissel, S. Reijm, G.M.C. Janssen, P.A. van Veelen, H. Bang, T.M.W. Huizinga, L.A. Trouw, D. van der Woude, R.E.M. Toes.
Annals of the Rheumatic Diseases. 2019 Jul;78(7):908-916

Autoantibody status is not associated with early treatment response to first-line methotrexate in patients with early rheumatoid arthritis.
J.S. Dekkers, S.A. Bergstra, A. Chopra, M. Tikly, J.E. Fonseca, K. Salomon-Escoto, T.W. Huizinga, D. van der Woude.
Rheumatology (Oxford). 2019 Jan 1;58(1):149-153.

***Aggregatibacter actinomycetemcomitans*–induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis**
M. Volkov, J.S. Dekkers, B.G. Loos, S. Bizzarro, T.W.J. Huizinga, H.A. Praetorius, R.E.M. Toes, D. van der Woude.
Science Translational Medicine. 2018 Mar 21;10(433).

Breach of autoreactive B-cell tolerance by post-translationally modified foreign proteins.
J.S. Dekkers, M.K. Verheul, J.N. Stoop, B. Liu, A. Ioan-Facsinay, P.A. van Veelen, A.H. de Ru, G.M.C. Janssen, M.Hegen, S.Rapecki, T.W. J. Huizinga, L.A. Trouw, R.E.M. Toes.
Annals of the Rheumatic Diseases. 2017 Aug;76(8):1449-1457.

Possibilities for preventive treatment in rheumatoid arthritis? Lessons from experimental animal models of arthritis: a systematic literature review and meta-analysis.
Dekkers J.S., Schoones J.W., Huizinga T.W., Toes R.E.M., van der Helm-van Mil A.H.
Annals of the Rheumatic Diseases. 2017 Feb;76(2):458-467.

The role of anticitrullinated protein antibodies in the early stages of rheumatoid arthritis.
Dekkers J, Toes RE, Huizinga TW, van der Woude D.
Current Opinion in Rheumatology. 2016 May;(3):275-81.

Tumour-derived exosomes as antigen delivery carriers in dendritic cell-based immunotherapy for malignant mesothelioma.
Mahaweni N.M., Kaijen-Lambers M.E., Dekkers J., Aerts J.G., Hegmans J.P.
Journal of Extracellular Vesicles. 2013 Oct 24;2.

Curriculum Vitae

Jacqueline Stephanie Dekkers werd op 3 mei 1989 geboren in Rotterdam. In 2007 behaalde zij haar vwo diploma op het Emmaus College te Rotterdam en startte zij met haar studie geneeskunde aan het Erasmus Medisch Centrum. In 2008 begon zij met haar tweejarige research master Molecular Medicine naast haar studie geneeskunde in Rotterdam. Voor haar research master heeft zij twee stages gedaan bij de afdeling Longziekten in het Erasmus MC en bij de afdeling Oncologie van het Gustave Roussy Instituut in Parijs. Voor haar onderzoeksproject ontving zij de prestigieuze KNAW young scientist grant en de Descartes Bourse d'excellence. Na het behalen van haar arts-examen in 2014 startte zij met haar promotieonderzoek op de afdeling Reumatologie van het LUMC onder begeleiding van prof.dr. R.E.M. Toes en prof.dr. T.W.J. Huizinga. Dit onderzoek richtte zich op de rol van auto-immuniteit en het ontstaan van auto-antistoffen in reumatoïde artritis. In september 2017 begon zij aan haar medische specialisatie tot reumatoloog. Momenteel is zij werkzaam bij de afdeling Interne Geneeskunde te Gouda.

Dankwoord

Voor het voltooien van mijn proefschrift heb ik van velen steun en hulp gekregen. In mijn dankwoord wil ik graag gebruik maken om jullie hiervoor persoonlijk te bedanken.

Allereerst wil ik iedereen op de afdeling Reumatologie bedanken voor de directe en indirecte bijdrage en met name het werkplezier en de goede sfeer op de afdeling. De afdeling reumatologie in het LUMC bestaat uit een prachtige groep collegae en ik heb het ontzettend naar mijn zin gehad tijdens mijn promotieonderzoek.

Mijn promotor, professor Toes, beste René dank voor alle adviezen, begeleiding en de leerzame besprekingen. Jouw kritische blik voor de juiste studie opzet van experimenten en het schrijven van wetenschappelijke artikelen is ontzettend waardevol. Promotor professor Huizinga, beste Tom, jouw enthousiasme en passie om basaal onderzoek en de kliniek samen te brengen is een enorme inspiratie.

Daarnaast wil ook graag Diane en Leendert bedanken voor hun steun en input in onze wetenschappelijke projecten. Diane, jij en Sytske-Anne hebben mij veel geleerd van de statistiek en onderzoeksmethoden, bedankt voor jullie hulp. Leendert, ik wil je graag bedanken voor al je enthousiasme voor het anti-CarP project. Je staat altijd open voor een nieuwe hypothese maar je blijft ook kritisch kijken naar de data.

Verder wil ik heel graag al mijn collega promovendi van het lab en het klinisch onderzoek bedanken. Mijn collega's van het anti-CarP team; Marije, Myrthe, Rosalie, Jeroen en Nivine, bedankt voor alle inbreng en de wetenschappelijke discussies. Sanne, Hester, Daniël, Mikhail, Jeroen en Ellen bedankt voor de hulp met de experimenten en de tijd samen met mij in het PDC.

Lise, Hilde, Joost, Rochelle, Corrie, Hendy, Linda, Uli en Andrea bedankt voor de wetenschappelijke discussies en de gezelligheid! Sanne, Marije en Mikhail, bedankt voor de samenwerking met de experimenten en het schrijven van de artikelen. We kunnen samen trots zijn op het resultaat.

Verder wil ik graag alle andere mede-promovendi bedanken; Sytske Anne, Joy, Debbie, Emma, Robin, Pauline, Anoek, Queeny, Sjoerd, Wendy, Leonie, Xanthe, Aleid, Wouter en Leonie. Bedankt voor de goede samenwerking en gezelligheid. Het is ontzettend leuk dat sommigen van jullie nog steeds mijn directe collega's zijn in Gouda!

Mijn lieve paranimfen Ilona en Femke, bedankt voor jullie hulp bij het voltooien van mijn boekje en tijdens mijn verdediging. Bedankt voor jullie enthousiasme.

Lieve Patricia, Roland en Ilona, ik wil jullie bedanken voor jullie onvoorwaardelijke steun. Bedankt voor jullie adviezen en alle aandacht die jullie mij geven. Dankzij jullie heb ik het doorzettingsvermogen en de inspiratie gekregen om deze uitdaging aan te gaan.

Tot slot, lieve Raoul, wil ik jou bedanken voor al je liefde, steun en interesse in alles wat ik doe. We kunnen samen trots zijn op het mooie resultaat. Ik wil je bedanken voor al onze mooie momenten samen en ik kan niet wachten op onze nieuwe avonturen samen in de toekomst!