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TRANSLATIONAL SCIENCE

Different classes of anti-modified protein antibodies are induced on exposure to antigens expressing only one type of modification

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ABSTRACT

Objectives Autoantibodies against post-translationally modified proteins (anti-modified protein antibodies or AMPAs) 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, there is no conceptual framework explaining the concurrent presence or mutual relationship of different AMPA responses in RA. Here, we aimed to gain understanding of the co-occurrence of AMPA by postulating that the AMPA response shares a common 'background' that can evolve into different classes of AMPAs.

Methods Mice were immunised with modified antigens and analysed for AMPA responses. In addition, reactivity of AMPA purified from patients with RA towards differently modified antigens was determined.

Results Immunisation with carbamylated proteins induced AMPAs recognising carbamylated proteins and also acetylated proteins. Similarly, acetylated proteins generated (autoreactive) AMPAs against other modifications as well. Analysis of anti-citrullinated protein antibodies from patients with RA revealed that these also display reactivity to acetylated and carbamylated antigens. Similarly, anti-carbamylated protein antibodies showed cross-reactivity against all three post-translational modifications.

Conclusions 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 the disease.

Key messages

What is already known about this subject?

- Antibodies targeting different post-translational modified proteins have been described for patients with rheumatoid arthritis (RA). Different classes of these antibodies can be present simultaneously. Nevertheless, the mechanisms behind the concurrent presence of different anti-modified protein antibody (AMPA) classes in RA are unclear.

What does this study add?

- Our data show that, in mice, a protein expressing one particular post-translational modification can induce cross-reactive AMPA against other post-translational modifications as well.
- Different AMPA from patients with RA show similar cross-reactivity.

How might this impact on clinical practice or future developments?

- Our results indicate a 'common' B-cell response from which different AMPA responses originate, thereby providing a conceptual framework for the mutual relationship and simultaneous presence of different AMPA 'classes' in RA.

INTRODUCTION

The presence of anti-citrullinated protein antibodies (ACPAs) is one of the hallmarks of rheumatoid arthritis (RA). ACPAs recognise citrullinated proteins and display an extensive citrulline-dependent cross-reactivity towards multiple citrullinated antigens.[1 2] 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.[3–7] Also, other post-translationally modified (PTM) proteins, in particular carbamylated and acetylated proteins, have been found to be recognised by RA autoantibodies.[8] Carbamylation and acetylation do not modify arginine, the target of citrullination, but lysine into respectively homocitrulline and acetyl-lysine. Homocitrulline is an amino acid resembling citrulline, but containing an additional methylene group. Anti-Carbamylated protein (anti-CarP) antibodies are present in approximately 45% of patients with RA.[9] 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 patients with RA are positive for anti-CarP antibodies,

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indicating that these antibodies represent a different class of anti-modified protein antibodies.[9 10] Acetylation, on the other hand, is mediated by intracellular acetyltransferases. Anti-acetylated protein antibodies (AAPAs) are present in approximately 40% of patients with RA[11] and are mainly found in ACPA-positive RA, although also ACPA-negative patients with RA can be AAPA positive. Inhibition experiments showed limited cross-reactivity between anti-acetylated, anti-carbamylated and ACPAs, indicating that also AAPAs represent another class of anti-modified protein antibody (AMPA).[11]

These previous observations are interesting as they indicate that AMPAs, due to their concurrent presence in RA, have a commonality that is currently not understood. Here, we studied the possibility that the AMPA response originates from a common 'event' by analysing whether exposure to one particular class of modified proteins can generate different AMPA responses.

MATERIALS AND METHODS

Proteins, modifications and immunisations

All procedures for protein modification, mass spectrometry and immunisations are previously described and further detailed in the online supplementary materials.[9 12 13] Animal experiments were approved by the Ethical Committee for Animal Experimentation. All immunised mice were healthy and showed no signs of arthritis throughout the experiment.

Mass spectrometry

Procedure for the mass spectrometry analysis is described in detail in online supplementary materials and methods.

Detection of AMPAs

For the detection of AMPAs in mice, the following ELISA was performed: Modified proteins and their non-modified counterparts were coated at a concentration of 10 µg/mL in 0.1 M carbonate–bicarbonate buffer (pH 9.6) overnight on Nunc Maxisorp plates (Thermo Scientific). The plates were blocked with phosphate buffered saline (PBS)+1% bovine serum albumin. The mouse sera were diluted 1:100 in RIA buffer (10 mM Tris (pH 7.6), 350 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and incubated overnight. Binding of mouse IgG was detected with horseradish peroxidase–conjugated goat-anti-mouse IgG1 (cat. no. 1070-05; Southern Biotech) and subsequently visualised with ABTS. Washing steps were performed between each incubation with PBS+0.05% Tween 20. All incubations, aside from the incubations with goat-anti-mouse IgG1 and ABTS, were performed at 4°C, and the final two steps were performed at room temperature. Arbitrary units were calculated using a reference serum in serial dilution. The reference serum was acquired from CaOVA-immunised or Ac-OVA-immunised mice for the carbamylated or acetylated protein ELISA, respectively. For the inhibition experiments, the sera were pre-incubated with 0–0.2 mg/mL protein for 1 hour before transferring them to the ELISA plate.

Reactivity of purified ACPA and anti-CarP antibodies, obtained from sera and synovial fluid (SF) of patients with RA, was measured using modified vimentin peptides (plates and reagents were kindly provided by Orgentec), as previously described.[11] In addition, purified ACPA and anti-CarP antibodies were tested on CCP2 and Ca-FCS, respectively, according to protocols previously described.[9 14 15]

Patients with RA

The material of the ACPA-positive patients with RA was selected for ACPA purification based on the ACPA status and levels. The patients with RA fulfilled the European League Against Rheumatism/American College of Rheumatology 2010 classification criteria. Similar to the material from ACPA-positive patients, the material from anti-CarP-positive patients used for anti-CarP-antibody isolation was derived from patients screened for anti-CarP status and levels.

IgG-AMPA purification

Specific AMPAs are isolated as has been previously described for ACPA in Scherer *et al*.[16] In short, plasma or serum samples and SF were acquired from patients. The plasma, serum and SF samples were subsequently filtered (0.2 µM filters; Millipore) before purifying AMPA with affinity chromatography (ÄKTA; GE Healthcare). Purification was performed using HiTrap streptavidin HP 1 mL columns (GE Healthcare) coupled with biotinylated CCP2 peptides (obtained from J.W. Drijfhout, IHB LUMC) for the isolation of ACPA[17 18] 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 0.1 M glycine hydrogen chloride (HCl) pH 2.5 and neutralised with 2 M Tris. ACPA-$IgG_{1,2,4}$ was subsequently purified from ACPA with Prot A and Prot G HiTrap columns.

Statistics

Statistical tests were performed with Prism V.7 (GraphPad). Significance of AMPA reactivity on proteins was tested with paired t-test. Differences in titre were tested with Mann-Whitney U tests. Correlations were assessed with Spearman. A p value of <0.05 was considered significant.

RESULTS

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

To analyse whether AMPA recognising different classes of PTMs can be induced with an antigen expressing one defined modification, we immunised 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 and commercially available antibodies against either carbamylated or acetylated lysines in ELISA (online supplementary figure 1). 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 immunisation experiments as additional specificity control.

To discriminate between reactivity against the PTM and protein backbone used for immunisation, we employed modified fibrinogen (Fib) as read-out. In doing so, antibodies recognising OVA were not interfering with the detection of AMPA.[13] To control for possible baseline reactivity towards modified proteins, sera from non-immunised mice were taken along with the ELISA experiments. Indeed, no reactivity was observed to non-modified fibrinogen or its modified counterparts in naïve animals, indicating that without immunisations, AMPA responses are not present towards either modified fibrinogen (figure 1A) or mouse albumin (figure 2A).[13 19] Likewise, although a strong reaction against OVA was noted (data not

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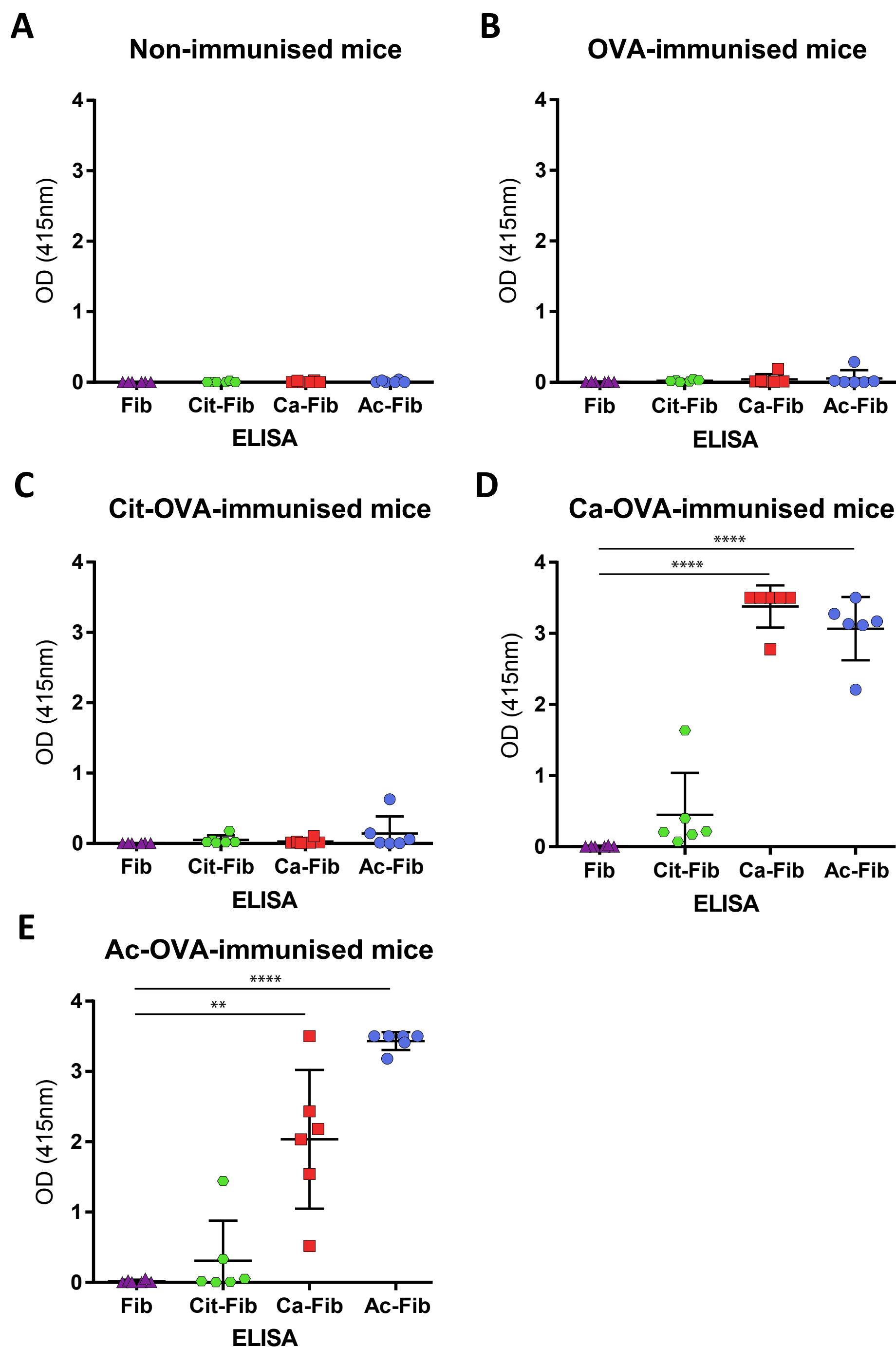

Figure 1 Immunisation with Ca-OVA or Ac-OVA induces antibody responses towards modified fibrinogen. Antibody reactivity towards modified fibrinogen in sera derived from non-immunised (A), OVA-immunised (B), Cit-OVA-immunised (C), Ca-OVA-immunised (D) or Ac-OVA-immunised (E) mice was measured by ELISA. Reactivity is depicted with OD values measured at 415 nm. For all groups, n=6. Representative data from two experiments is shown. Ac, acetylated; Ca, carbamylated; Cit, citrullinated; Fib, fibrinogen; OD, optical density; OVA, ovalbumin.

shown), indicating proper immunisation, mice immunised with non-modified OVA did not react to modified Fib (figure 1B) nor modified mouse albumin (figure 2B).[13] These results indicate that neither non-modified OVA nor the adjuvant used is driving AMPA production. We were unable to detect reactivity towards citrullinated Fib (Cit-Fib) in mice immunised with citrullinated OVA (Cit-Ova) (figure 1C). As ACPAs have been reported in some murine models,[20–23] we additionally tested the sera on modified myelin basic protein (MBP), but again we were unable to detect citrulline reactivity (online supplementary figure 2). Mice immunised with carbamylated OVA (Ca-OVA), however, displayed a strong reactivity towards Ca-Fib, but not non-modified-Fib (figure 1D). Remarkably, sera of mice immunised with Ca-OVA also reacted to Ac-Fib and to some extent to Cit-Fib. This reactivity was further validated using modified MBP (online supplementary figure 2). Moreover, these sera also reacted to both Ac-mouse albumin (Ac-mAlb) and Ca-mAlb (figure 2C), indicating that exposure to modified foreign proteins is capable of inducing a breach of tolerance towards self-antigens carrying different classes of modifications. These data are intriguing as they indicate that antibody responses induced by carbamylated antigens are able

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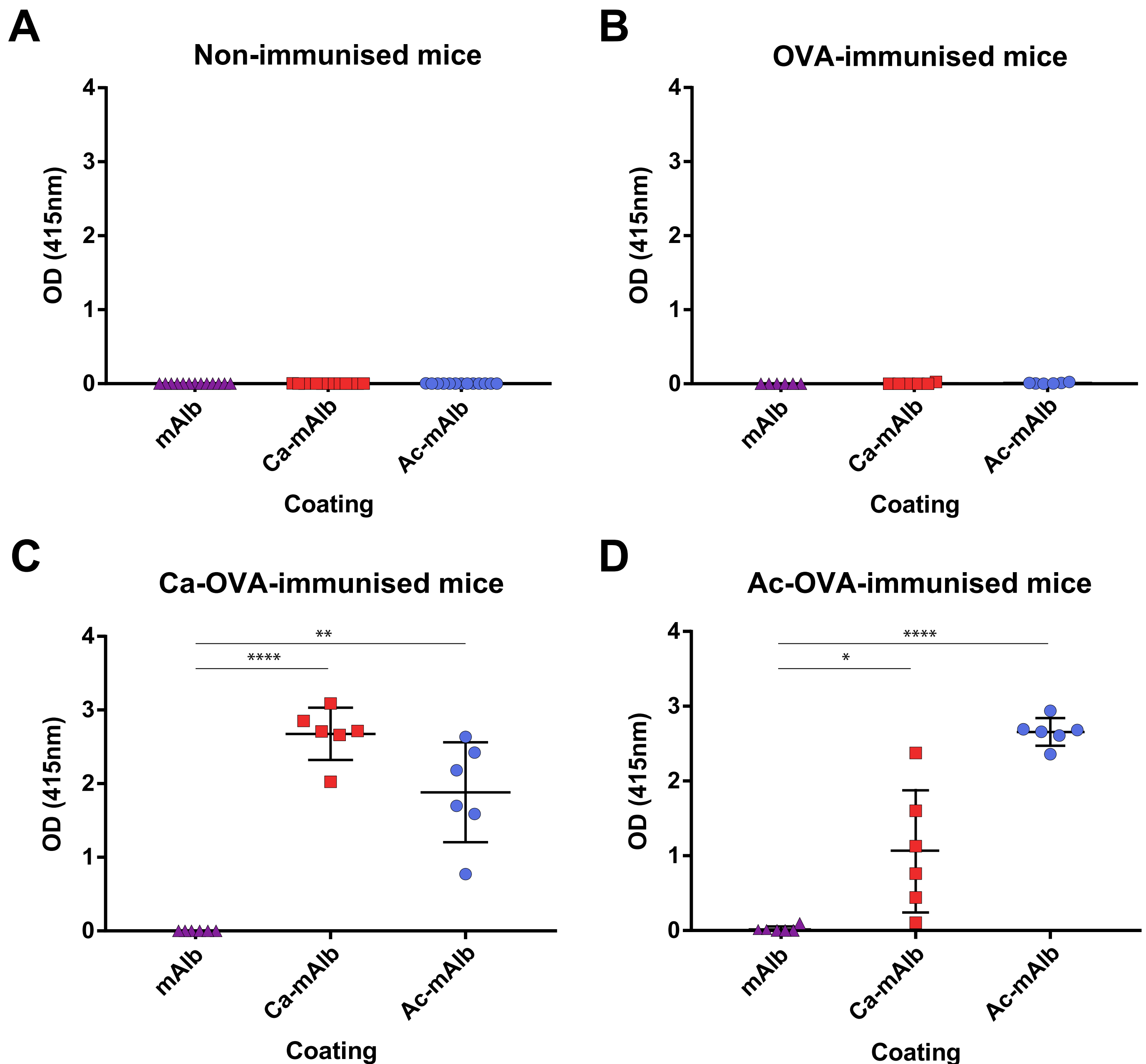

Figure 2 Break of tolerance towards modified self-proteins in Ca-OVA-immunised and Ac-OVA-immunised mice. Reactivity towards carbamylated and acetylated mouse albumin was tested by ELISA (A) with sera derived from non-immunised (A), OVA-immunised (B), Ca-OVA-immunised (C) and Ac-OVA-immunised (D) mice. Results show representative data from two immunisation experiments. $p<0.05$ depicts significance. Ac, acetylated; Ca, carbamylated; OD, optical density; OVA, ovalbumin

to recognise multiple modifications, pointing to the generation of cross-reactive (autoreactive) AMPAs induced by exposure to only one class of modified antigen.

Next, we wished to determine whether cross-reactive antibodies could also be induced by immunisation with acetylated OVA. We observed reactivity to Ac-Fib as expected and also towards Ca-Fib (figure 1E). Reactivity towards Cit-Fib was only moderately apparent. This could not be validated using Cit-MBP (online supplementary figure 2). Similar reactivity patterns were observed when modified mouse albumin was used as model autoantigen (figure 2D). Together, these results indicate that immunisation with Ac-OVA induces (auto-)antibodies cross-reactive to acetyl-lysine and homocitrulline.

Cross-reactive antibody responses harbour different PTM recognition profiles

To further investigate the cross-reactive nature of these AMPA responses in more detail, we next analysed the autoantibody titre through dilution of sera from immunised animals. Similar antibody titres were observed towards Ac-Fib and Ca-Fib in Ca-immunised mice (figure 3A). In contrast, the titre of antibodies recognising Ac-Fib was considerable higher than the antibody titre against Ca-Fib in Ac-OVA-immunised mice (figure 3B). These data indicate that in contrast to anti-CarP antibodies in Ca-OVA-immunised mice, the AAPA response in Ac-OVA-immunised mice is only partly cross-reactive to both modifications.

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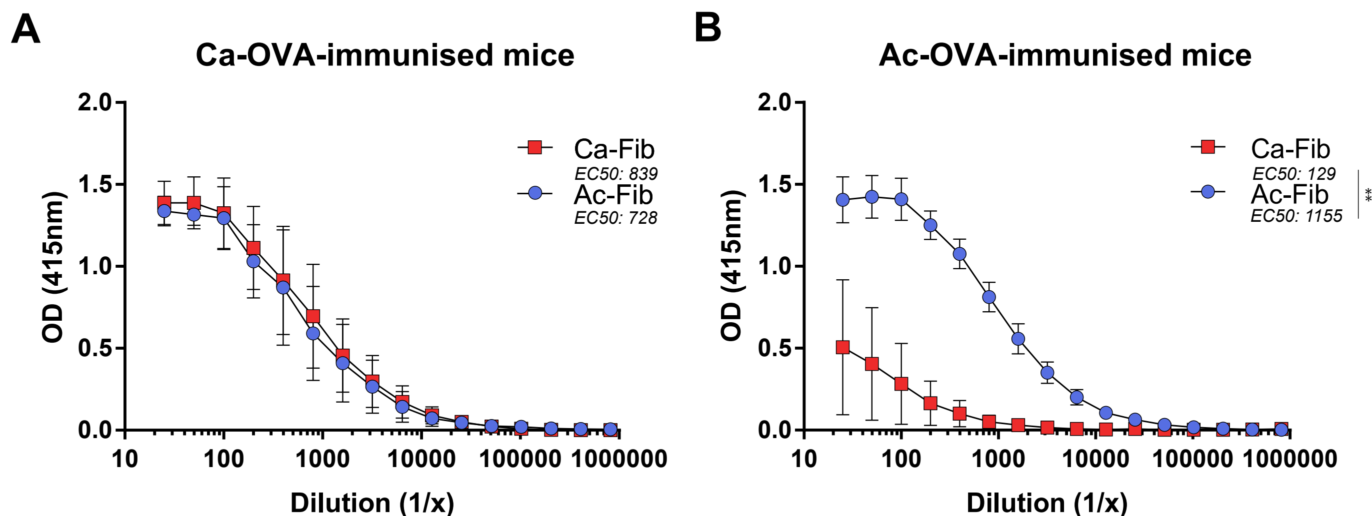

Figure 3 Antibody titres and avidity in sera of Ca-OVA-immunised and Ac-OVA-immunised mice. Antibody titres as measured by ELISA on Ca-Fib and Ac-Fib for Ca-OVA-immunised (A) and Ac-OVA-immunised (B) mice. EC50 depicts the dilution at which half of the maximum effective reactivity is present. Representative data from two immunisation experiments are shown. Ac, acetylated; Ca, carbamylated; Fib, fibrinogen; EC50, effective concentration at 50%; OD, optical density; OVA, ovalbumin.

The data presented on antibody titre also predict that the AMPA response present in Ca-OVA-immunised mice (highly cross-reactive) can be readily inhibited by both acetylated and carbamylated proteins, whereas the AMPA reaction in Ac-OVA-immunised mice can only be fully inhibited by acetylated proteins. To confirm this notion, the binding capacity towards Ca-Fib and Ac-Fib was analysed by inhibition experiments with modified fibrinogen. Indeed, for Ca-OVA-immunised mice, antibody reactivity towards either modified antigen could be inhibited by Ac-Fib (figure 4A, B), whereas for Ac-OVA-immunised mice, Ac-Fib reactivity could not be inhibited by competing with Ca-Fib (figure 4C, D). These data confirm that the AMPA response generated by Ca-OVA-immunisation is highly cross-reactive, whereas part of the antibodies induced by Ac-OVA immunisation are cross-reactive towards both modifications.

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

The data presented above show that exposure of mice to a protein carrying one defined PTM can induce cross-reactive AMPAs. To address whether also in humans AMPA are cross-reactive towards different classes of modified antigens, we next isolated ACPA-IgG from SF or plasma of seven patients with RA as previously described.[17 18] We focused on ACPA as the ACPA response is the most prominent AMPA response in RA. As depicted in figure 5A, B, ACPA-IgG were strongly enriched following isolation, whereas the flow-through contained low to no levels of ACPA-IgG (online supplementary figure 3). Next, the purified ACPA-IgG were analysed 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 patients with RA are cross-reactive towards carbamylated antigens as observed previously[9] and that they can also recognise acetylated antigens. To analyse whether also anti-CarP antibodies display cross-reactivity towards different classes of PTMs, we next isolated anti-CarP antibodies from sera of two anti-CarP-positive patients. As shown in figure 5C, 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 the three 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.

DISCUSSION

RA is characterised by the presence of autoantibodies against different PTMs, including citrullinated, carbamylated and acetylated proteins. As different AMPAs target different PTMs and are generally seen as distinct autoantibody families, it is intriguing that their presence often goes together in RA. At present, there is no conceptual framework explaining the concurrent presence of different AMPA responses in RA. Here, we show that exposure to a protein carrying one defined PTM can lead to cross-reactive (auto)antibody responses towards different PTMs. Interestingly, we have shown that AMPA from patients with RA purified with antigens carrying one particular PTM can recognise different classes of PTMs too, indicating a cross-reactive nature of these autoantibodies as well. These findings are important as they indicate that the different AMPA responses observed in RA can potentially be generated by antigen(s) carrying only one particular modification. Similarly, they provide a rationale for the simultaneous manifestation of multiple AMPA reactivities in RA.

Given the observations that different AMPAs target different antigens and are generally seen as distinct autoantibody 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 represent 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 recognising 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.[24] Our data indicate that exposure to a defined antigen displaying a particular class of

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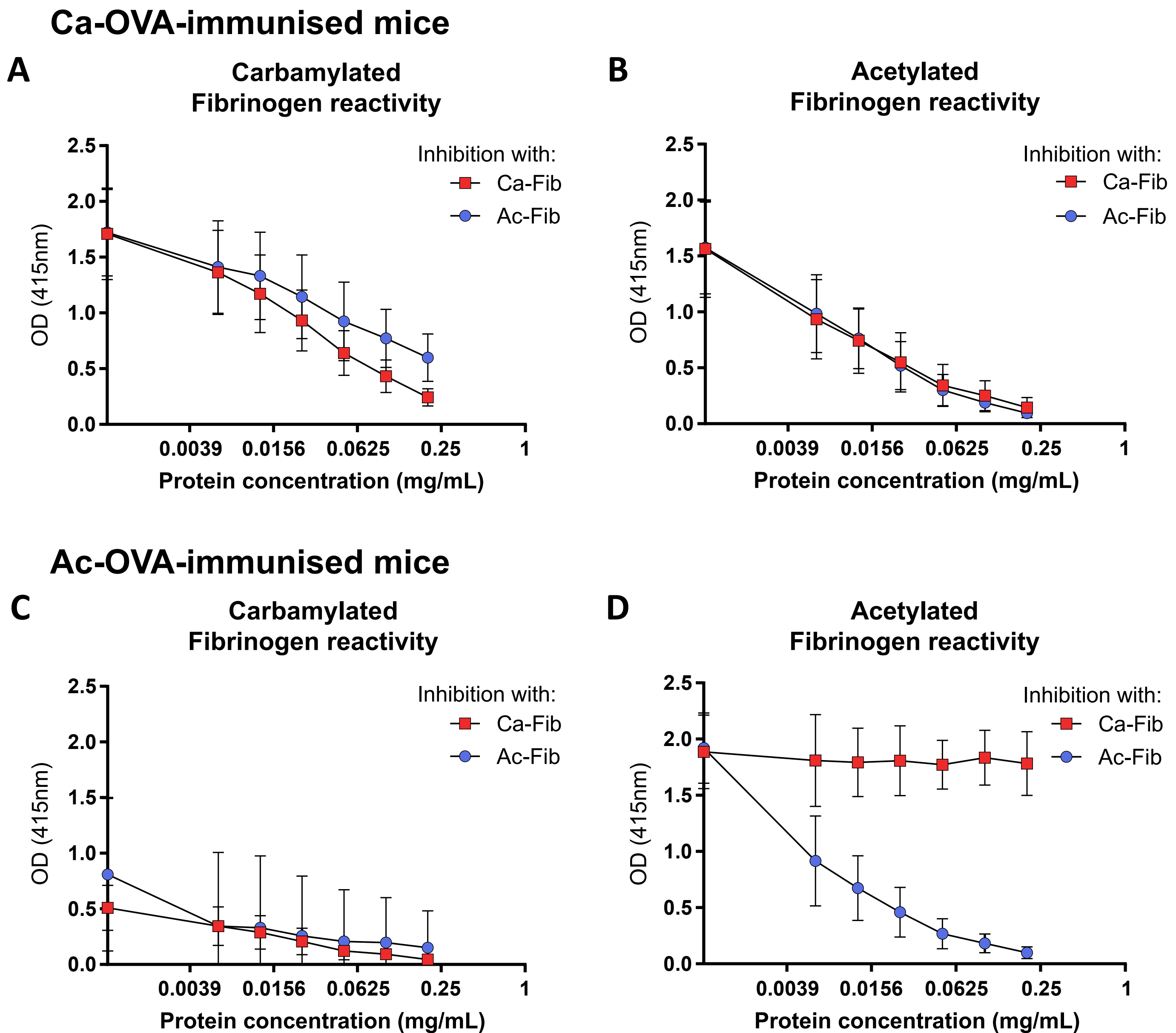

Figure 4 Inhibition of antibody binding by pre-incubation of mouse sera with modified fibrinogen. Cross-reactivity of antibodies is studied by assessment of the inhibitory capacity of pre-incubating sera with modified fibrinogen. Sera from Ca-OVA-immunised mice were pre-incubated with varying concentrations of modified fibrinogen before testing the antibody reactivity on Ca-Fib (A) or Ac-Fib (B). Sera from Ac-OVA-immunised mice were pre-incubated with varying concentrations of modified fibrinogen before testing the antibody reactivity on Ca-Fib (C) or Ac-Fib (D). Results show representative data of two experiments. Ac, acetylated; Ca, carbamylated; Fib, fibrinogen; OD, optical density; OVA, ovalbumin.

PTM can lead to a cross-reactive antibody response recognising several classes of modified antigens, conceivably explaining the co-occurrence of multiple AMPA reactivities in RA.

It has been shown that ACPA and anti-CarP antibodies can be cross-reactive towards citrullinated and carbamylated antigens.[9] Citrulline and homocitrulline are highly similar in structure as they differ in only one methyl group, even though they are conversions from different amino acids. We now show that also acetylated antigens can be recognised by these antibodies. This was unexpected as acetyl-lysine shares less structural homology to citrulline/homocitrulline (online supplementary figure 1). The cross-reactivity towards acetylated antigens was even more prominent in mice because AMPA induced by Ca-OVA immunisation did not recognise citrullinated proteins, even though they are able to recognise acetylated lysines.

The finding that exposure to, for example, an acetylated protein leads to the formation of autoantibodies 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, for example, ACPA or anti-CarP antibodies does not have to be citrullinated or carbamylated, but could be represented by, for example, an acetylated protein. Clearly, at present, we cannot conclude from our data whether a particular PTM antigen initiates AMPA induction in RA. Nonetheless, it will be relevant to study in pre-disease samples whether a breach of tolerance towards, for example, acetylated or carbamylated proteins precedes ACPA production or vice versa and whether this is similar in all patients or can vary from patient to patient.

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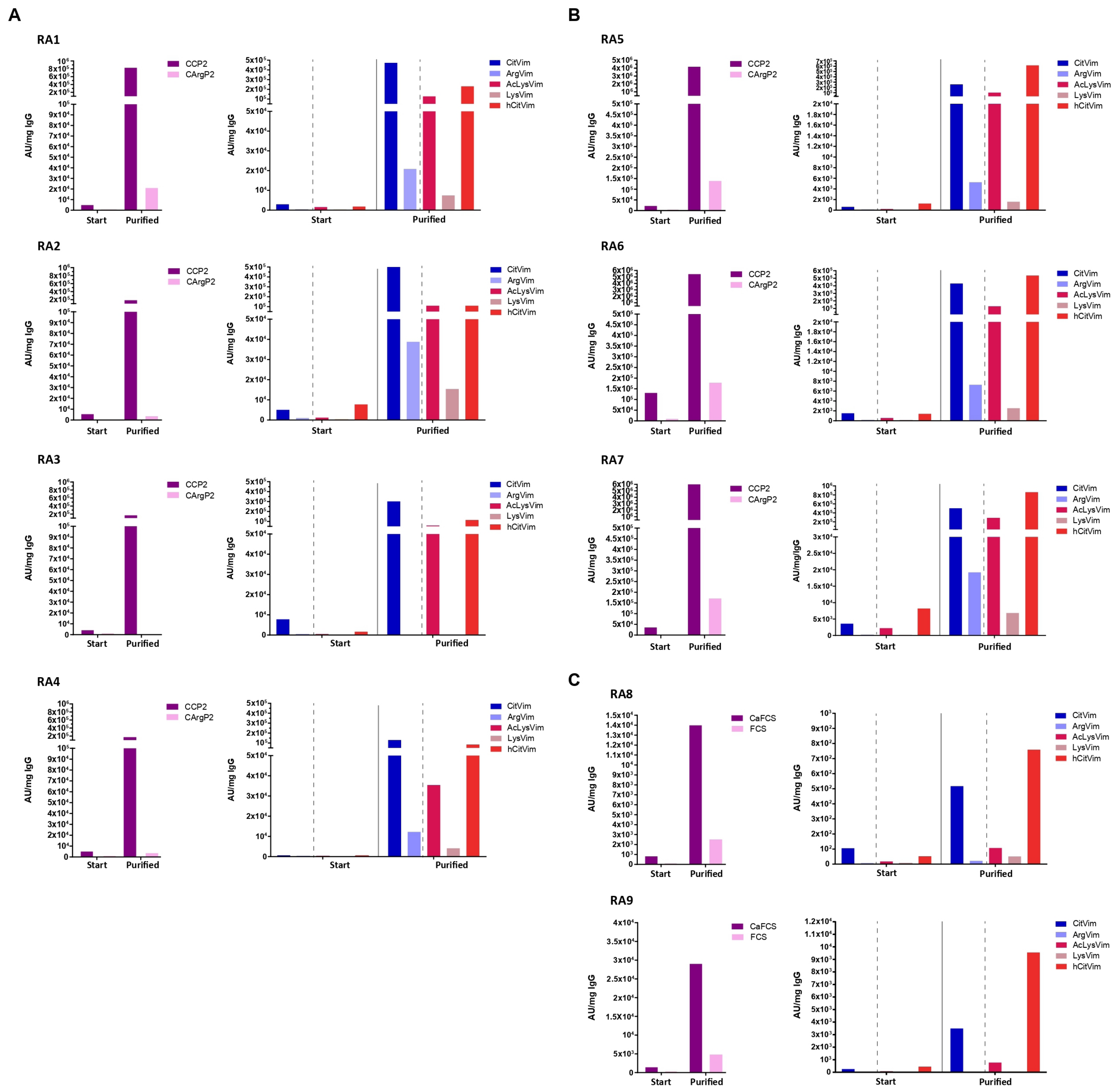

Figure 5 Cross-reactivity of purified human ACPA or anti-CarP antibodies towards modified vimentin peptides. ACPA and anti-CarP antibodies were isolated from patients with rheumatoid arthritis (RA). ACPA from synovial fluid (A, n=4) and serum (B, n=3) from patients were tested on CCP2 and modified vimentin peptides. Anti-CarP antibodies from serum of patients with RA (c, n=2) were tested on Ca-FCS and modified vimentin peptides. Reactivity is depicted as arbitrary units per milligram IgG and calculated based on standards. AcLys, acetylated lysine; Arg, arginine control; AU/mg IgG, arbitrary units per milligram immunoglobulin G; Ca, carbamylated; CArgP2, cyclic arginine control peptide; CCP2, cyclic citrullinated peptide; Cit, citrullinated; FCS, fetal calf serum; hCit, homocitrulline (carbamylated); Lys, lysine control; Vim, vimentin peptide.

An increasing number of studies suggest that mucosal surfaces, specifically the periodontium, the gut and the lungs, could be sites of disease initiation of RA and indicate the microbiome as an important driver of the initiation of autoimmunity. In this respect, especially protein acetylation by bacteria might now also be incriminated in the induction of autoantibody responses against PTM proteins. Recent evidence shows that many bacterial species are able to acetylate proteins,[25] including bacteria proposed as link between periodontal infection and RA.[26] Given our observation that AMPAs recognising 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 autoimmunity in RA. Therefore, a logical next step is to test faecal extracts from patients with RA also 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 (eg, through infection) could lead to the formation of acetylated proteins detected

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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. In this scenario, it is conceivable that microbe-specific T cells help the B cell initially recognising the microbe-derived modified protein. On further somatic hypermutation, the B-cell response could be selected/start recognising other modified proteins explaining the cross-reactive nature of AMPAs and the observation that different AMPAs often appear together in patients. Likewise, the diversification of an initial AMPA response towards other PTMs could, potentially, also explain the observation that the HLA-shared-epitope (SE) alleles are associated with ACPA-positive RA, whereas the first appearance of ACPA in healthy subjects is HLA-SE-allele independent.[27 28] Possibly, by diversification towards citrulline recognition, an initially HLA-SE-independent AMPA reaction against, for example, acetylated proteins could recruit new HLA-SE-restricted T cells required for further broadening of the AMPA/ACPA response associated with disease precipitation. Thus, in this scenario, the link to the microbiome, the cross-reactive nature of AMPAs, the breach of tolerance to modified self-proteins, the HLA-SE association with the 'second hit', as well as the concurrent presence 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 immunising 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. Furthermore, we would like to emphasise that, despite the advantages of using a controlled setting for the immunisation of mice, a major pitfall of studying RA-associated antibodies in mice is the inability to induce detectable production of ACPAs with our standard immunisation protocol, that is, 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. 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. Interestingly, recent studies have shown two monoclonal ACPAs able to interact with an acetylated histone peptide[29] as well as one able to recognise a carbamylated vimentin peptide.[30]

In conclusion, our data show that induction of cross-reactive AMPA can be achieved by the encounter with a protein carrying one specific PTM and indicate that the different AMPAs present in RA could have a common 'background', thereby providing novel insight into the concurrent presence of these antibodies in RA, an important hallmark of the disease.

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Contributors ASBK, JSD and RT have designed the experiments. ASBK and JSD have done the animal experiments. ASBK, JSD, MV and ALD have performed the ELISAs (murine and human). LH, ACK and MvD have performed the AMPA purification from patients with RA. GMCJ and PAvV have done the mass spectrometry analysis of the modified antigens. ASBK, JSD, MV, HB, TWJH, LAT, DvdW and RT were involved in critically revising the manuscript for intellectual improvement. ASBK, JSD, MV, ALD, TK, SR, LAT, DvdW and RT have been extensively involved in the interpretation and analysis of the results. All authors have contributed to the writing and editing of the manuscript.

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Competing interests None declared.

Patient consent for publication Obtained.

Ethics approval All animal experiments were approved by the Ethical Committee for Animal Experimentation of the LUMC, Leiden. The study with human material was conducted with the approval of the regional ethics committee at Leiden University Medical Center.

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Data sharing statement All data relevant to the study are included in the article or uploaded as supplementary information.

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