Tolerance and immune regulation in rheumatoid arthritis

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Chapter 5

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

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Adapted from: "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 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.

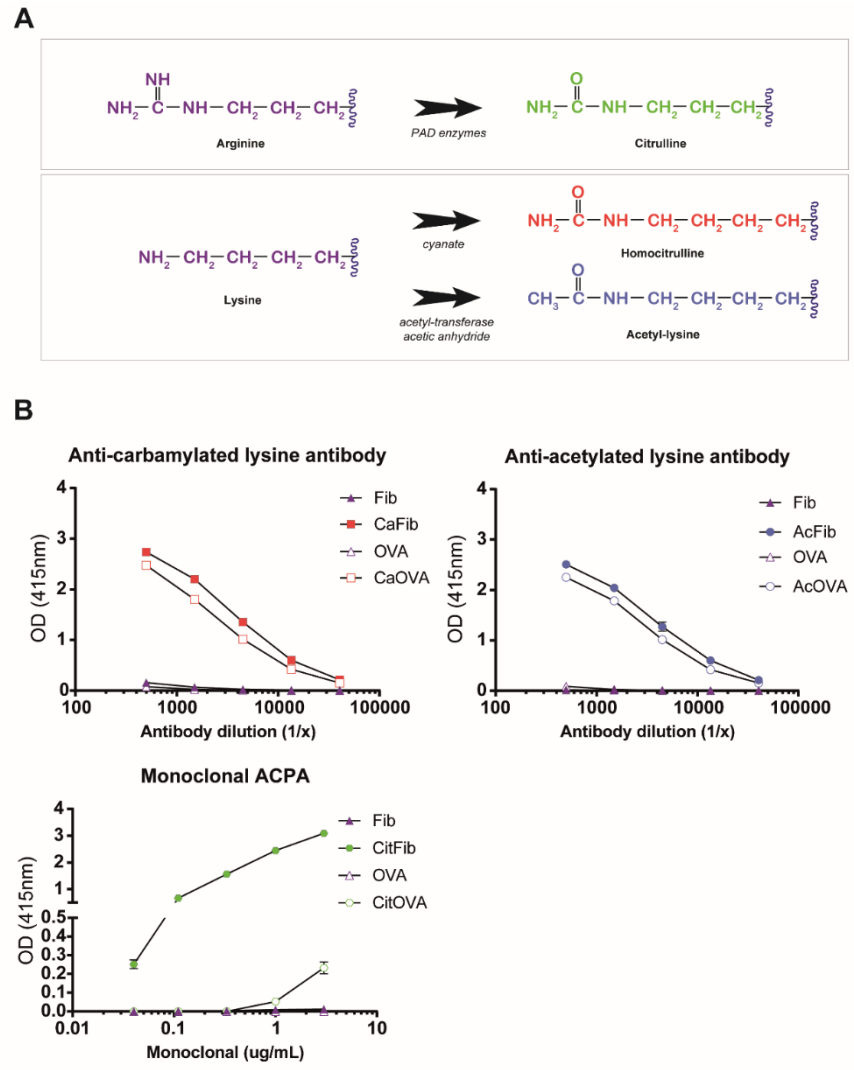


Figure 1. Structural overview of the posttranslational protein modifications. Schematic view of the amino acid structures of arginine and lysine, and their conversions towards citrulline, homocitrulline and acetylated lysine (A). ELISA with commercial polyclonal anti-acetylated-lysine antibodies, polyclonal 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.

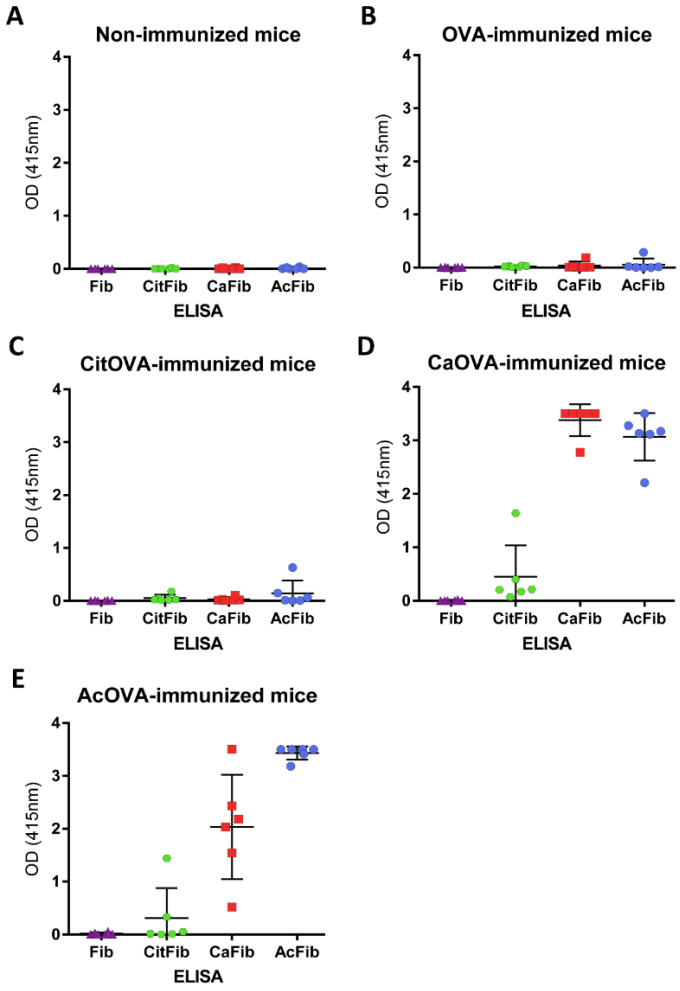


Figure 2. Immunization with CaOVA or AcOVA induces antibody responses towards modified fibrinogen. Immunization with Ca-OVA or Ac-OVA induces antibody responses towards modified fibrinogen. Antibody reactivity towards modified fibrinogen in sera derived from non-immunized (A), OVA-immunized (B), Cit-OVA-immunized (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.

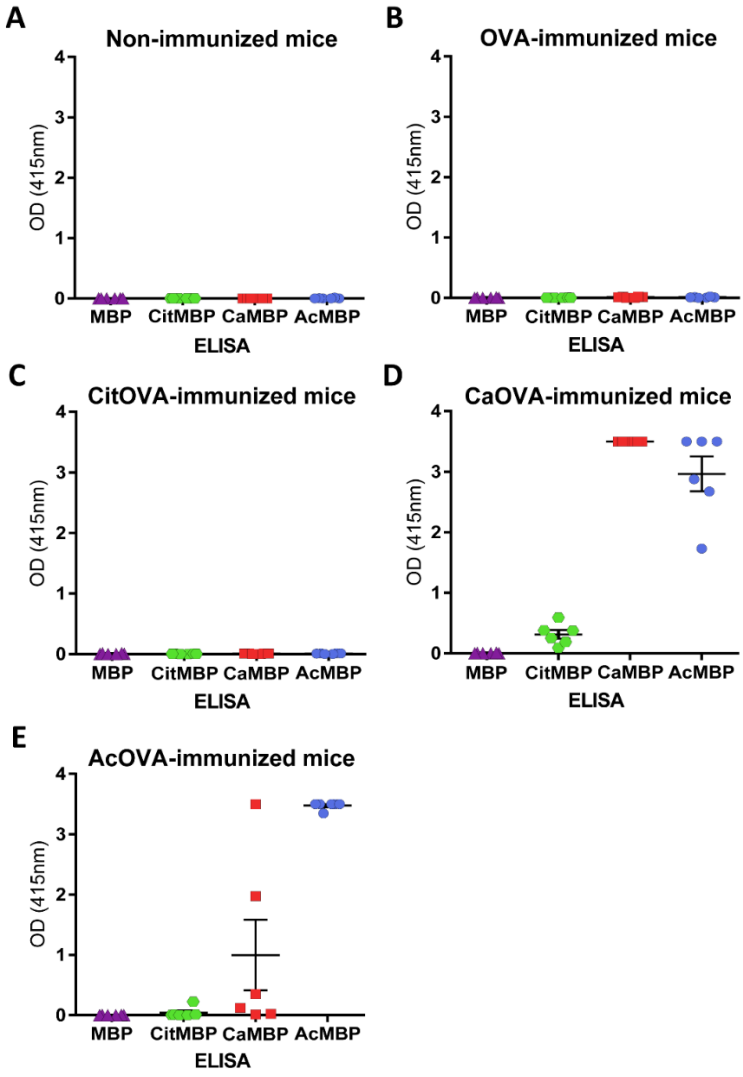


Figure 3. Immunization with CaOVA or AcOVA induces antibody responses towards modified MBP. Antibody reactivity towards modified MBP in sera derived from non-immunized (A), OVA-immunized (B), CitOVA-immunized (C), CaOVA-immunized (D) or AcOVA-immunized (E) mice was measured by ELISA. Reactivity is depicted 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.

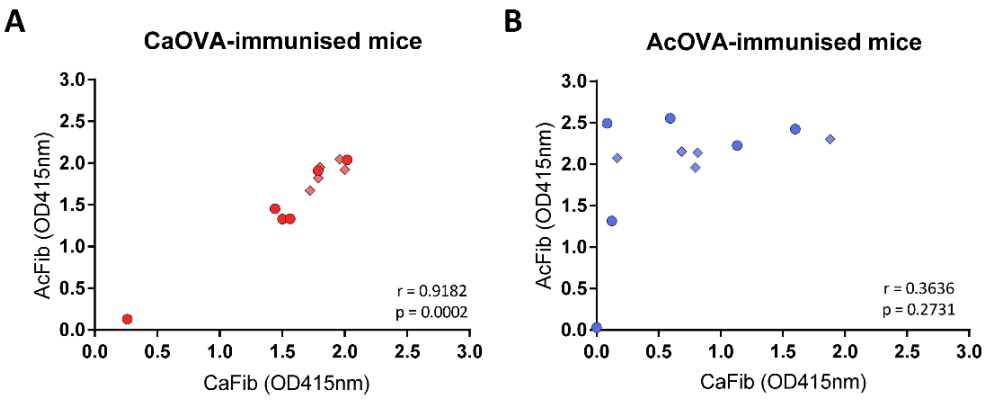


Figure 4. Correlation between CaFib- and AcFib-reactivity for CaOVA- and AcOVA-immunized mice. Reactivity towards CaFib and AcFib in sera from CaOVA- (A) and AcOVA-immunized (B) mice was measured with ELISA and correlated to each other. Data shown for two separate immunization experiments (diamonds (n=5) 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.

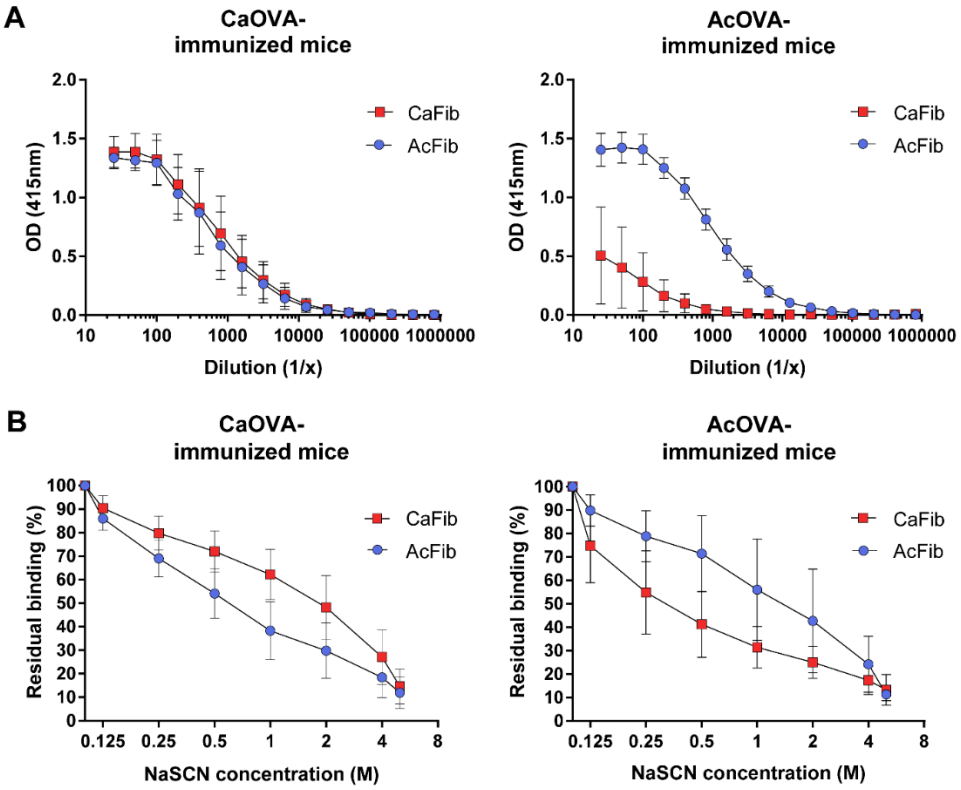


Figure 5. Antibody titers and avidity in sera of CaOVA- and AcOVA-immunized mice. Antibody titers as measured by ELISA on CaFib and AcFib for CaOVA- and AcOVA-immunized mice (A). IC50 depicts the dilution at which half of the highest reactivity is still visible. Representative data from two experiments 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.

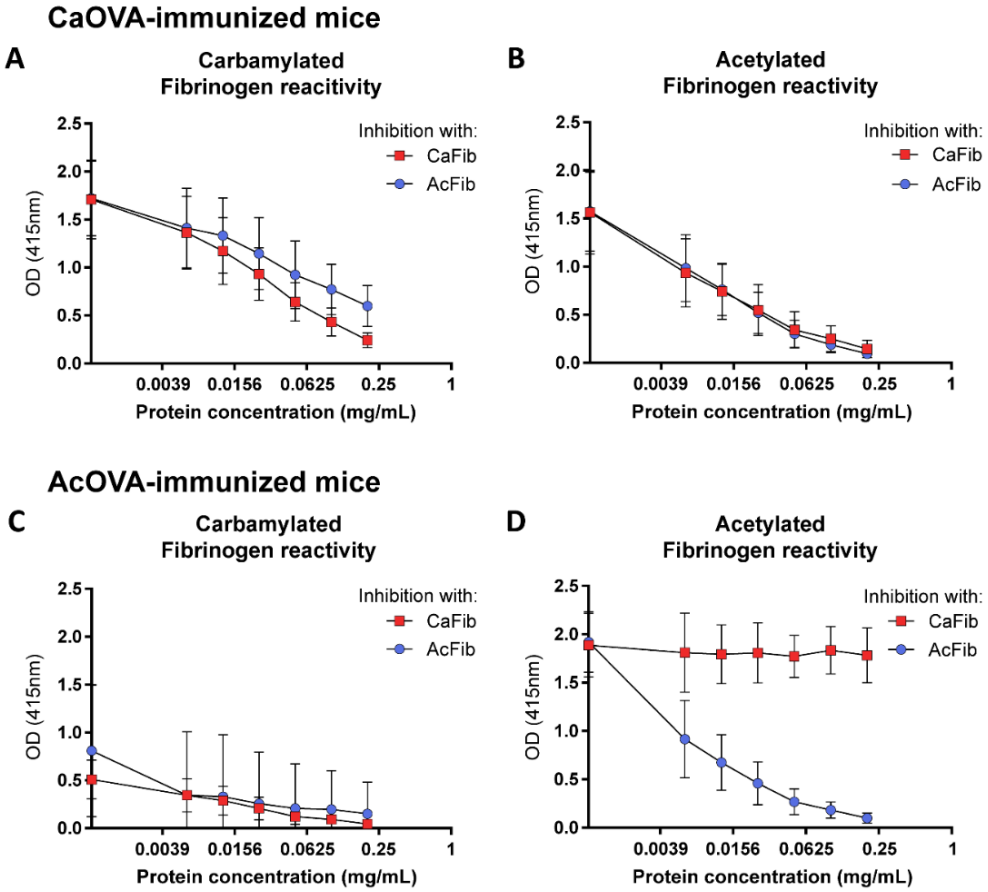


Figure 6. Inhibition of antibody binding by pre-incubation of mouse sera with modified fibrinogen. Cross-reactivity of antibodies is studied by assessment of the inhibitory capacity of pre-incubating sera with modified fibrinogen. Sera from CaOVA-immunized mice was pre-incubated with varying concentrations 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.

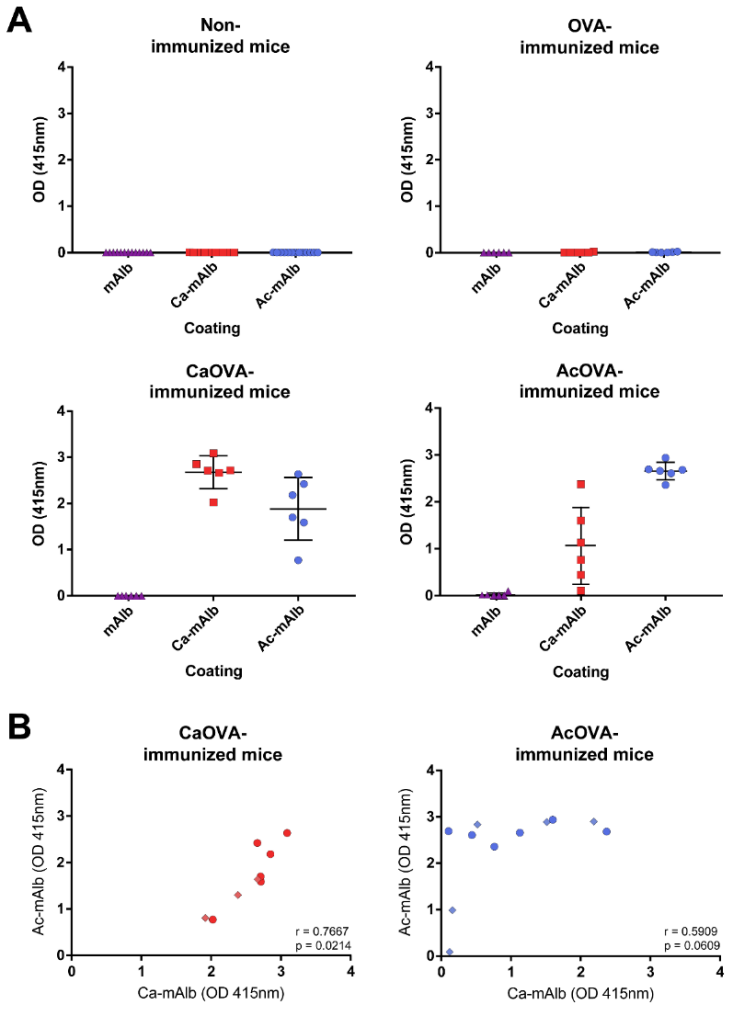
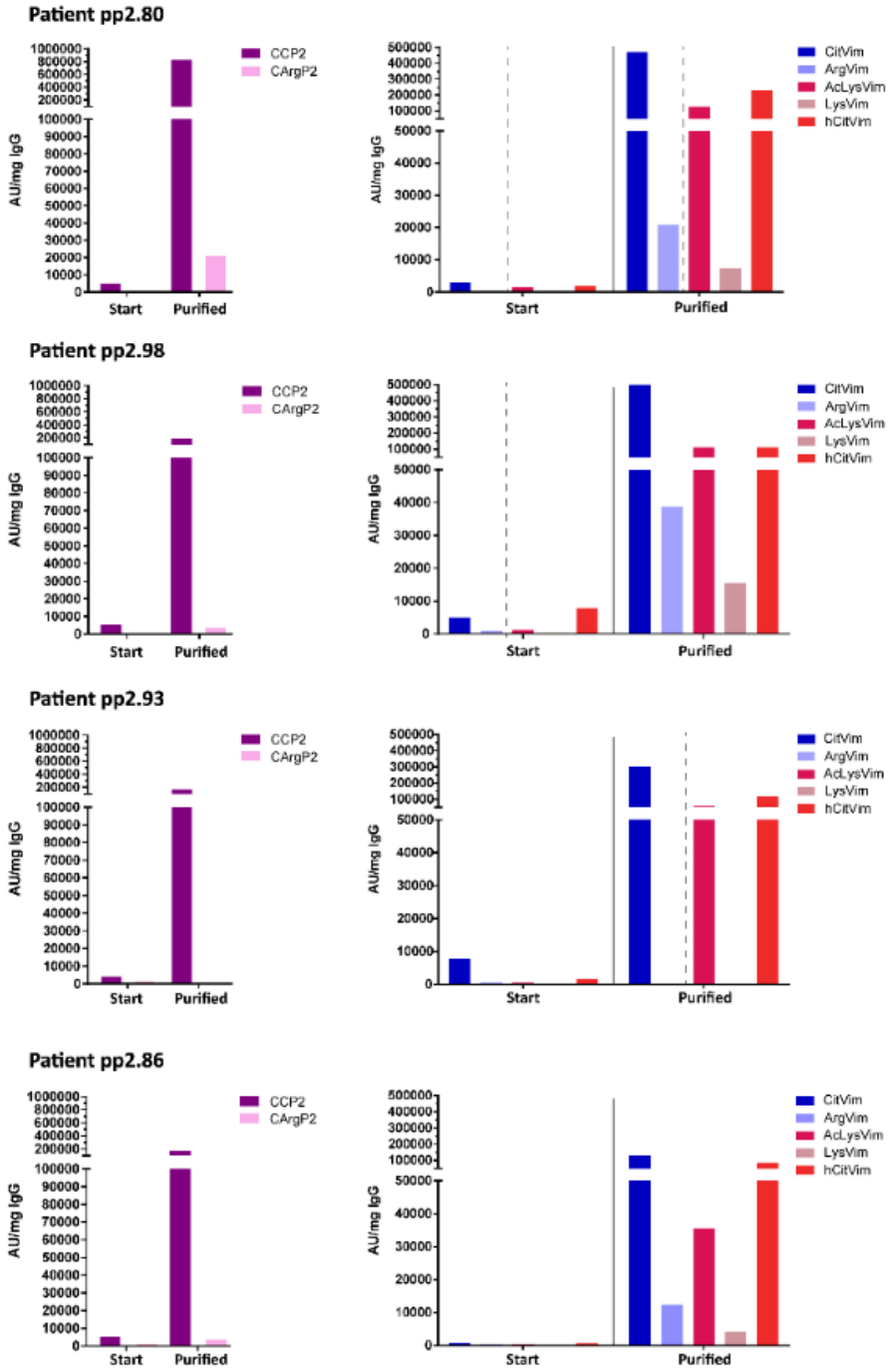


Figure 7. Break of tolerance towards modified self-proteins in CaOVA- and AcOVA-immunized mice. Reactivity in towards carbamylated and acetylated mouse albumin was tested by ELISA (A) with sera derived from non-immunized, OVA-, CaOVA- and AcOVA-immunized mice and shown with OD values. Results show representative 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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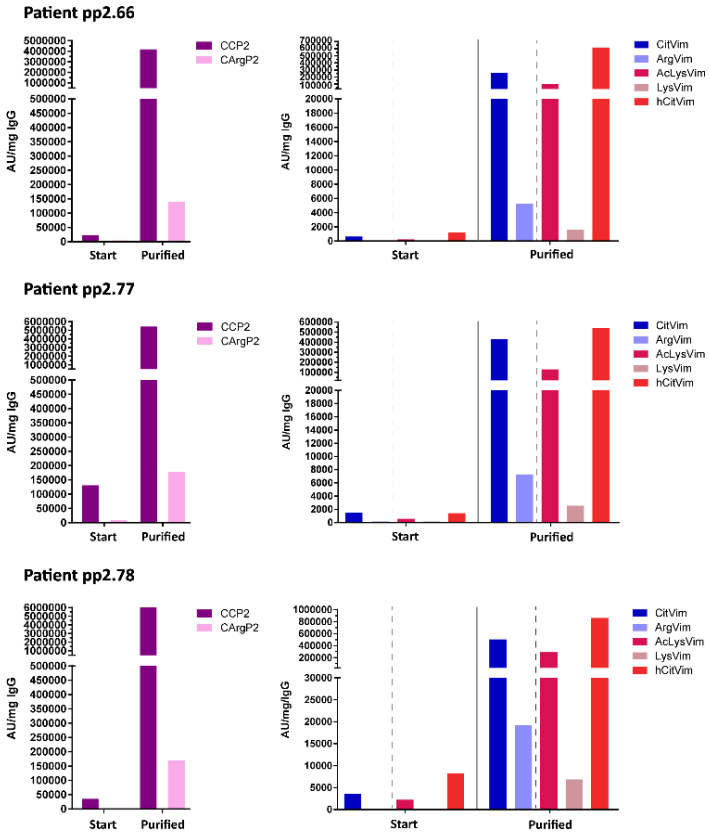
CCP2
CArgP2
AU/mg IgG
Start
Purified
CitVim
ArgVim
AcLysVim
LysVim
hCitVim
Patient pp2.98
Patient pp2.93
Patient pp2.86

B


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

C

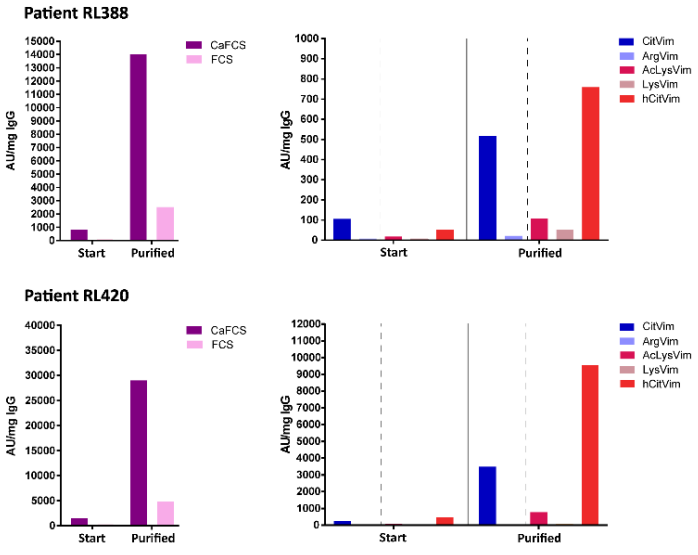
Patient RL388
CaFCS
FCS
AU/mg IgG
Start
Purified
CitVim
ArgVim
AcLysVim
LysVim
hCitVim
Patient RL420

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.

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