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Tolerance and immune regulation in rheumatoid arthritis
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Chapter 3

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

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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.

Chapter 3

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 $37^{\circ}C$

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 [TM] 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.
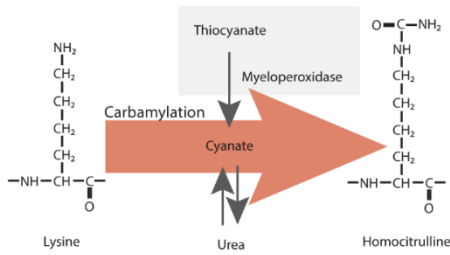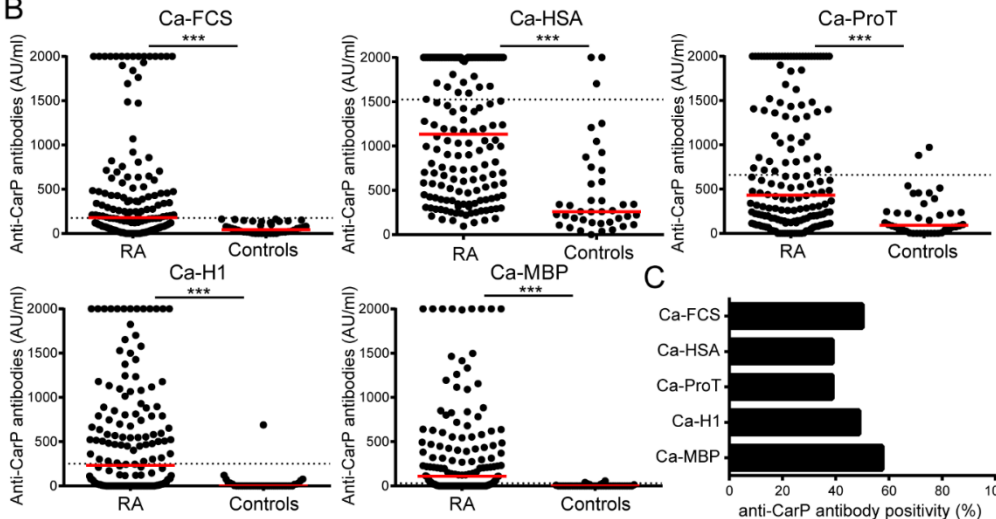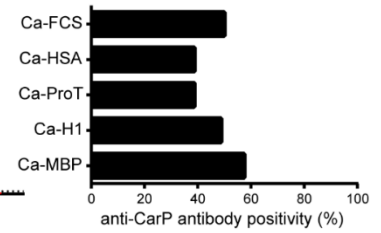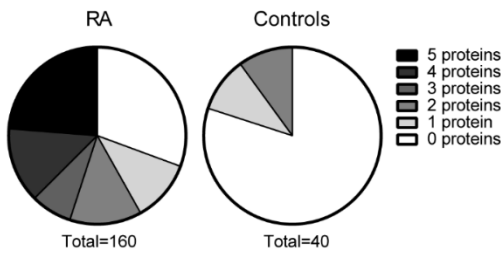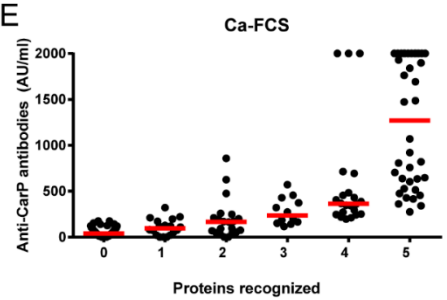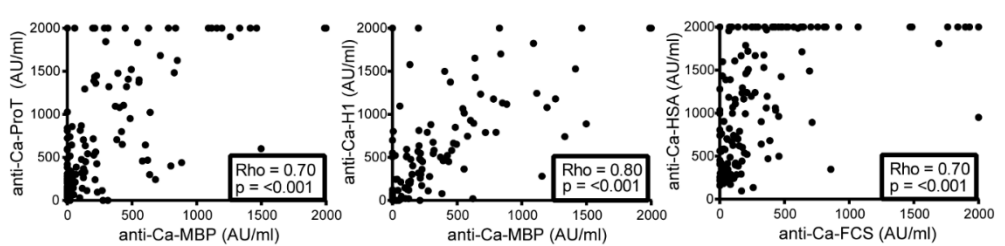
Figure 1. Characterization of anti-CarP antibody reactivities in RA patients. (A) During carbamylation a lysine residue is converted into a homocitrulline residue trough a chemical reaction with cyanate. Levels of cyanate are in equilibrium with urea and can be increased, for example, during kidney disease. 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.
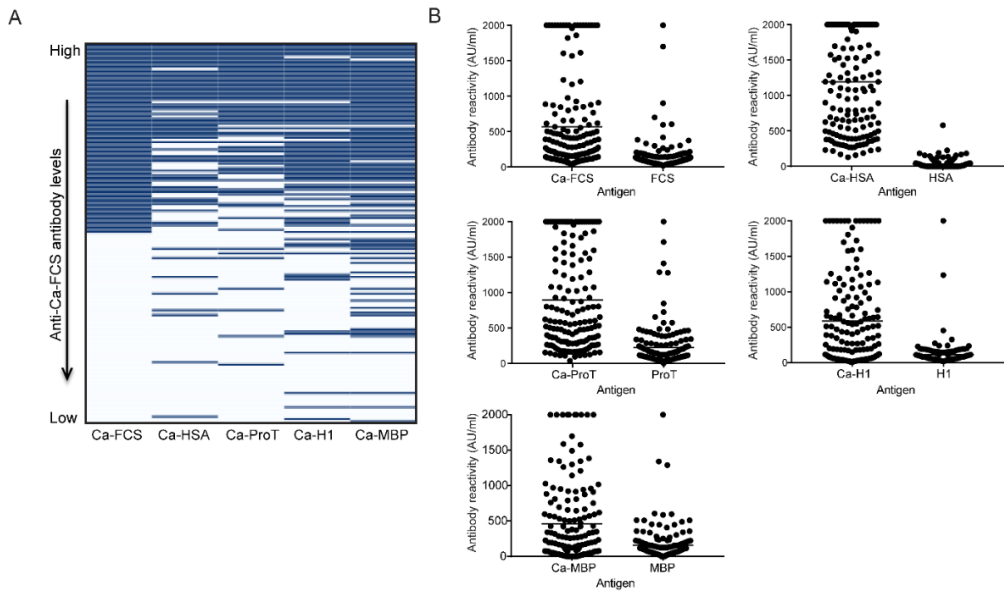
Figure 2. Anti-CarP antibodies of RA patients recognize multiple carbamylated antigens. (A) Anti-CarP antibody reactivities against five carbamylated and non modified counterparts were measured by ELISA. Differential binding of RA sera to the carbamylated proteins compared to the unmodified proteins 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.

Chapter 3

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.
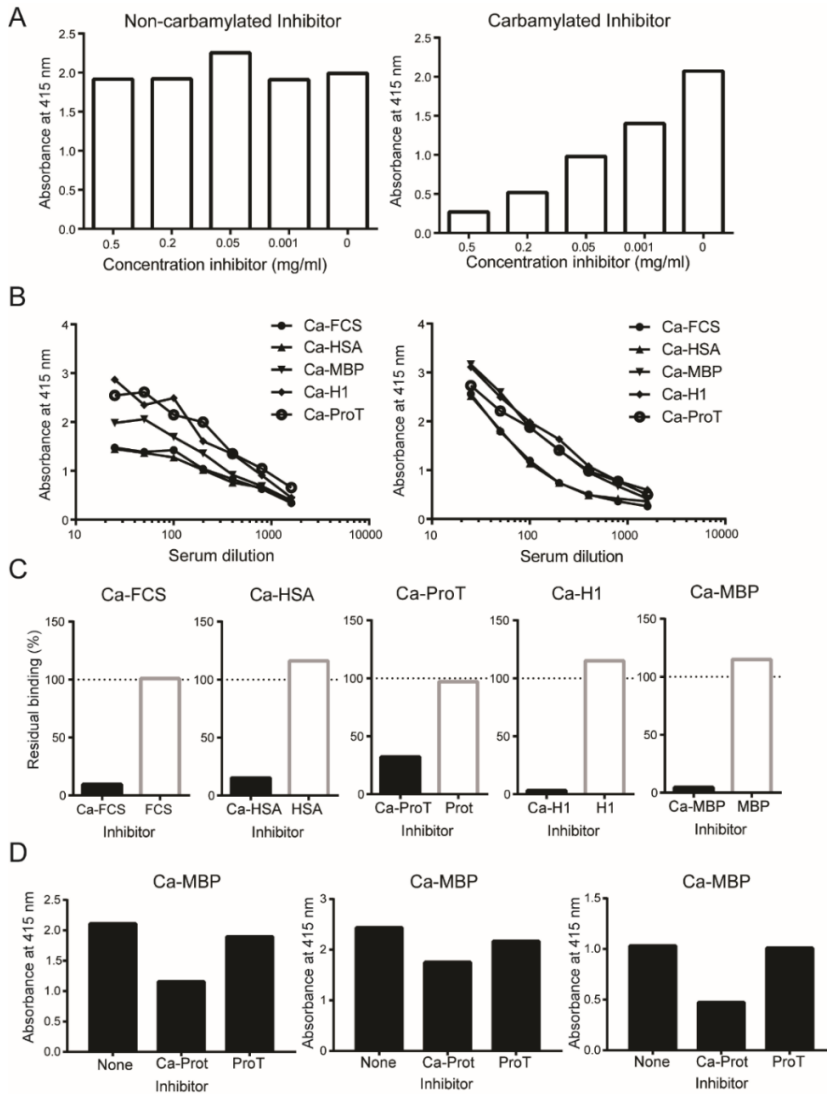
Figure 3. Titration and control experiments for the inhibition studies. (A) Representative figure of an inhibition assay in which the plate was coated with Ca-FCS and Ca-FCS (left) or FCS (right) was used as an inhibitor in different concentrations. (B) Titration assay of two RA serum samples containing 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.
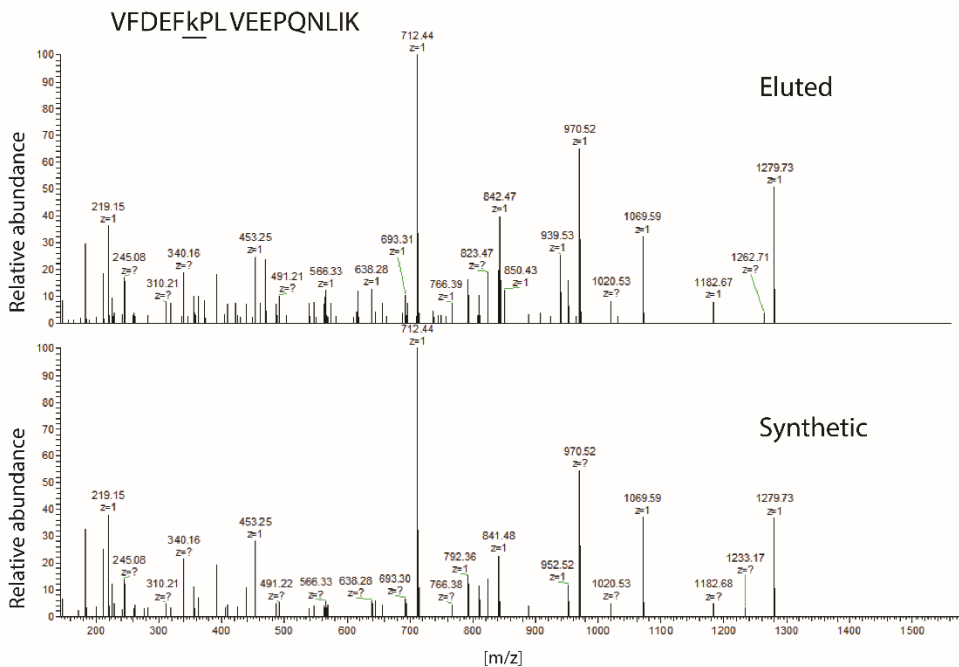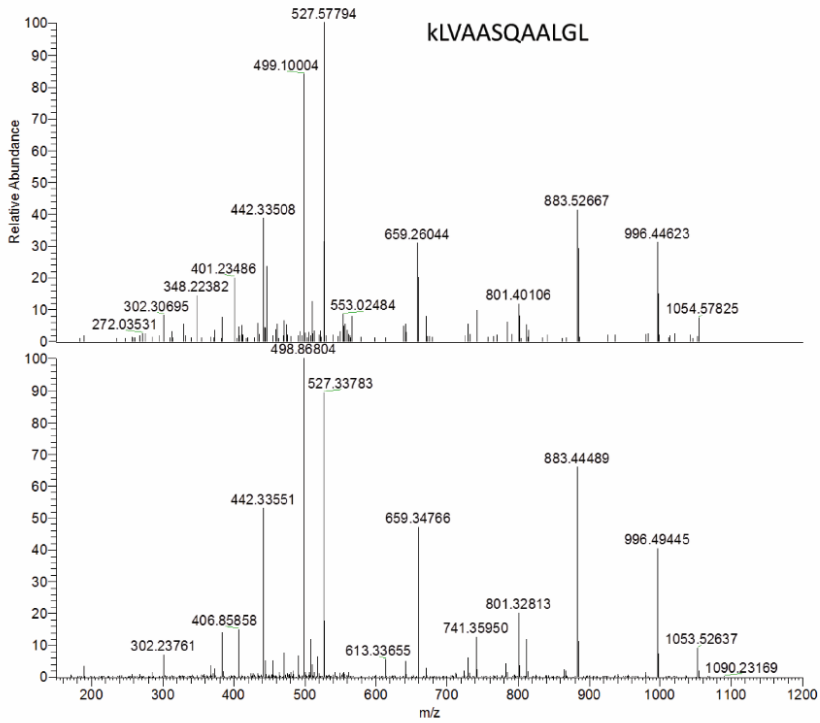
Figure 5. Identification of carbamylated albumin in RA synovial tissue. Tandem mass spectrometry (MS2) spectrum from eluted VFDEF**k**PLVEEPQNLIK peptide (upper panel) derived from carbamylated albumin identified in RA synovial tissue. The synthetic VFDEF**k**PLVEEPQNLIK peptide (lower panel) was submitted to MS2 on the same instrument. The bold, non-captical k, indicates the position of homocitrulline residue.

A — kLVAASQAALGL

B — kVPQVSTPTLVEVSR

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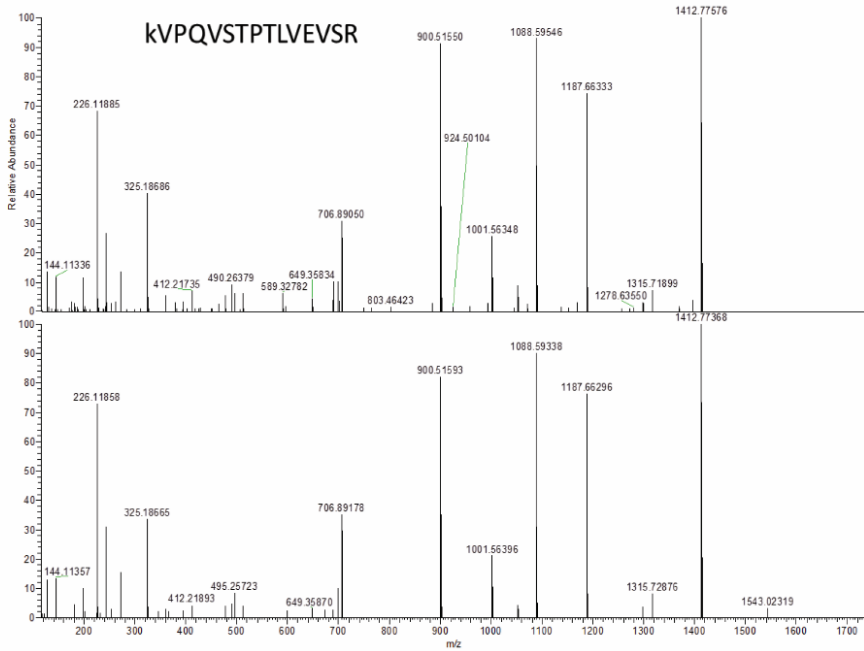
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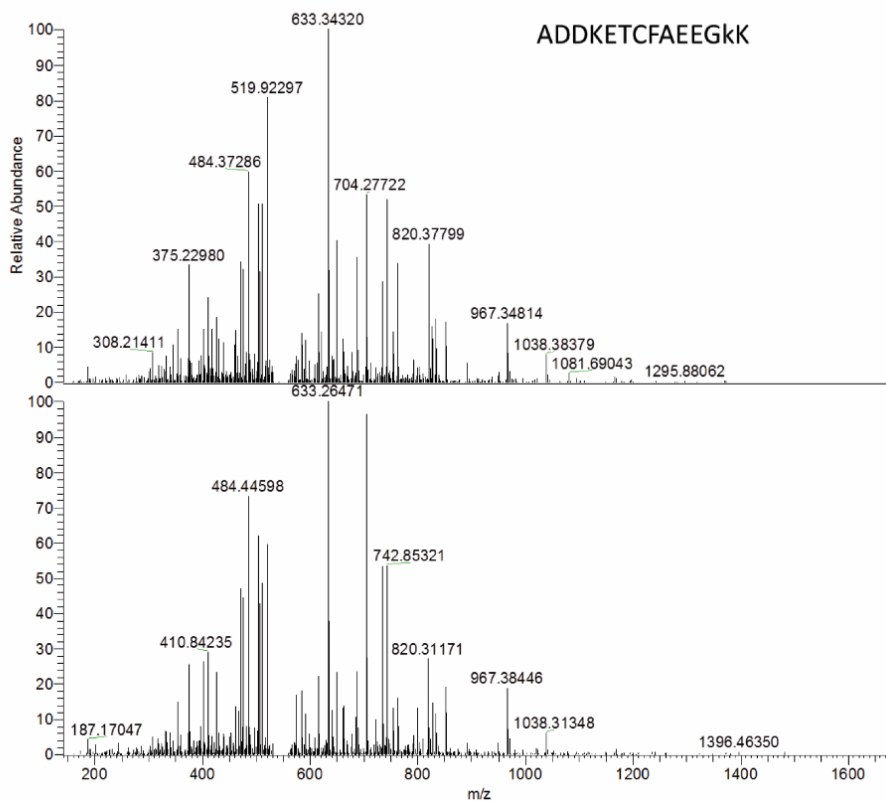
Figure 6. Additional carbamylated albumin peptides identified in the synovial compartment of RA patients. Additional peptides identified in the synovial compartment in RA. MS/MS spectra of peptides derived from carbamylated albumin identified in the RA synovial compartment (upper panel). The synthetic (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.
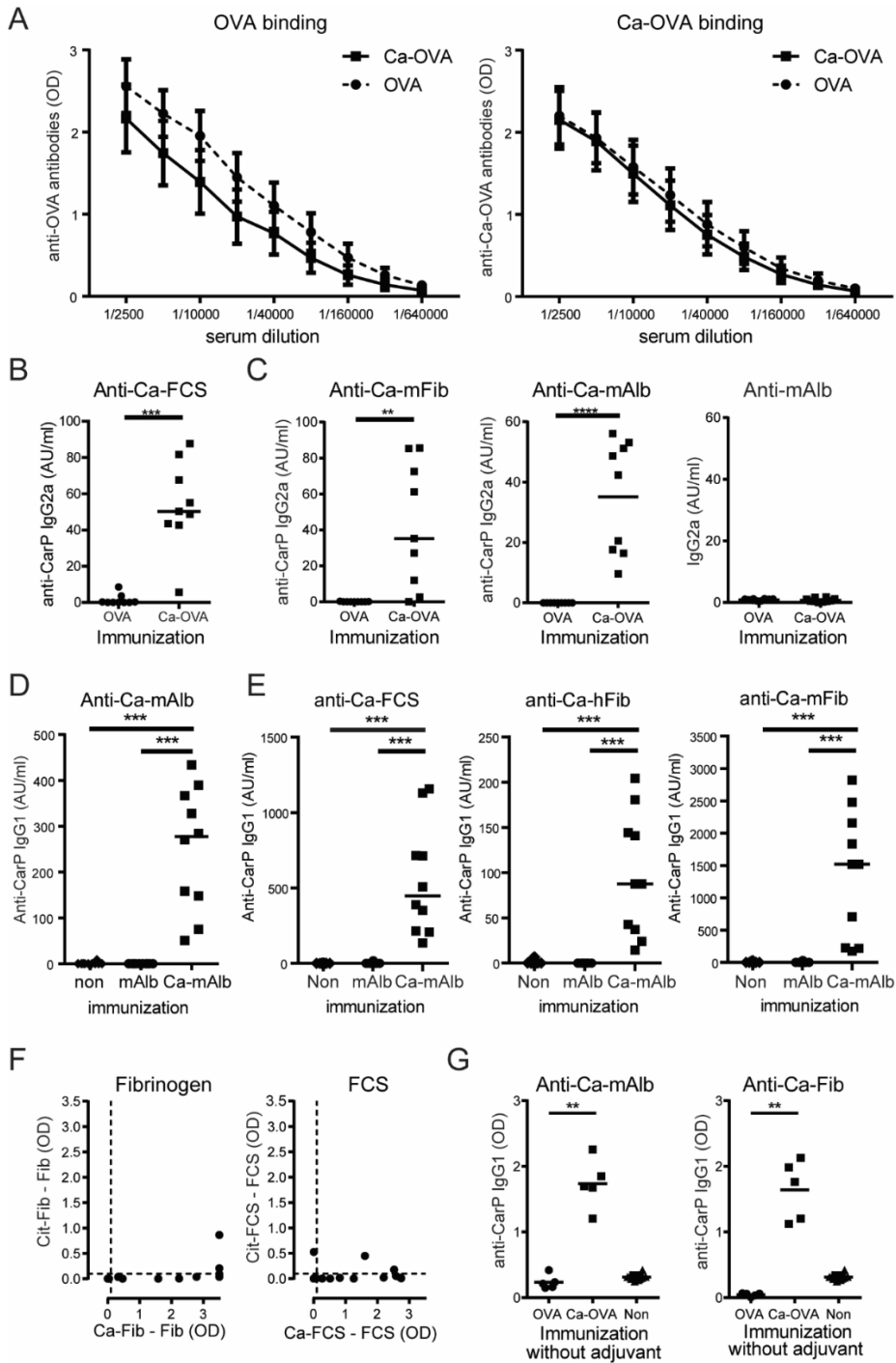
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A OVA binding

anti-OVA antibodies (OD)

serum dilution

■ Ca-OVA
● OVA

Ca-OVA binding

anti-Ca-OVA antibodies (OD)

serum dilution

■ Ca-OVA
● OVA

B Anti-Ca-FCS

anti-CarP IgG2a (AU/ml)

OVA   Ca-OVA
Immunization

***

C Anti-Ca-mFib

anti-CarP IgG2a (AU/ml)

OVA   Ca-OVA
Immunization

**

Anti-Ca-mAlb

anti-CarP IgG2a (AU/ml)

OVA   Ca-OVA
Immunization

****

Anti-mAlb

IgG2a (AU/ml)

OVA   Ca-OVA
Immunization

D Anti-Ca-mAlb

Anti-CarP IgG1 (AU/ml)

non   mAlb   Ca-mAlb
immunization

***
***

E anti-Ca-FCS

Anti-CarP IgG1 (AU/ml)

Non   mAlb   Ca-mAlb
immunization

***
***

anti-Ca-hFib

Anti-CarP IgG1 (AU/ml)

Non   mAlb   Ca-mAlb
immunization

***
***

anti-Ca-mFib

Anti-CarP IgG1 (AU/ml)

Non   mAlb   Ca-mAlb
immunization

***
***

F Fibrinogen

Cit-Fib - Fib (OD)

Ca-Fib - Fib (OD)

FCS

Cit-FCS - FCS (OD)

Ca-FCS - FCS (OD)

G Anti-Ca-mAlb

anti-CarP IgG1 (OD)

OVA   Ca-OVA   Non
Immunization
without adjuvant

**

Anti-Ca-Fib

anti-CarP IgG1 (OD)

OVA   Ca-OVA   Non
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, ** $p<0.01$).
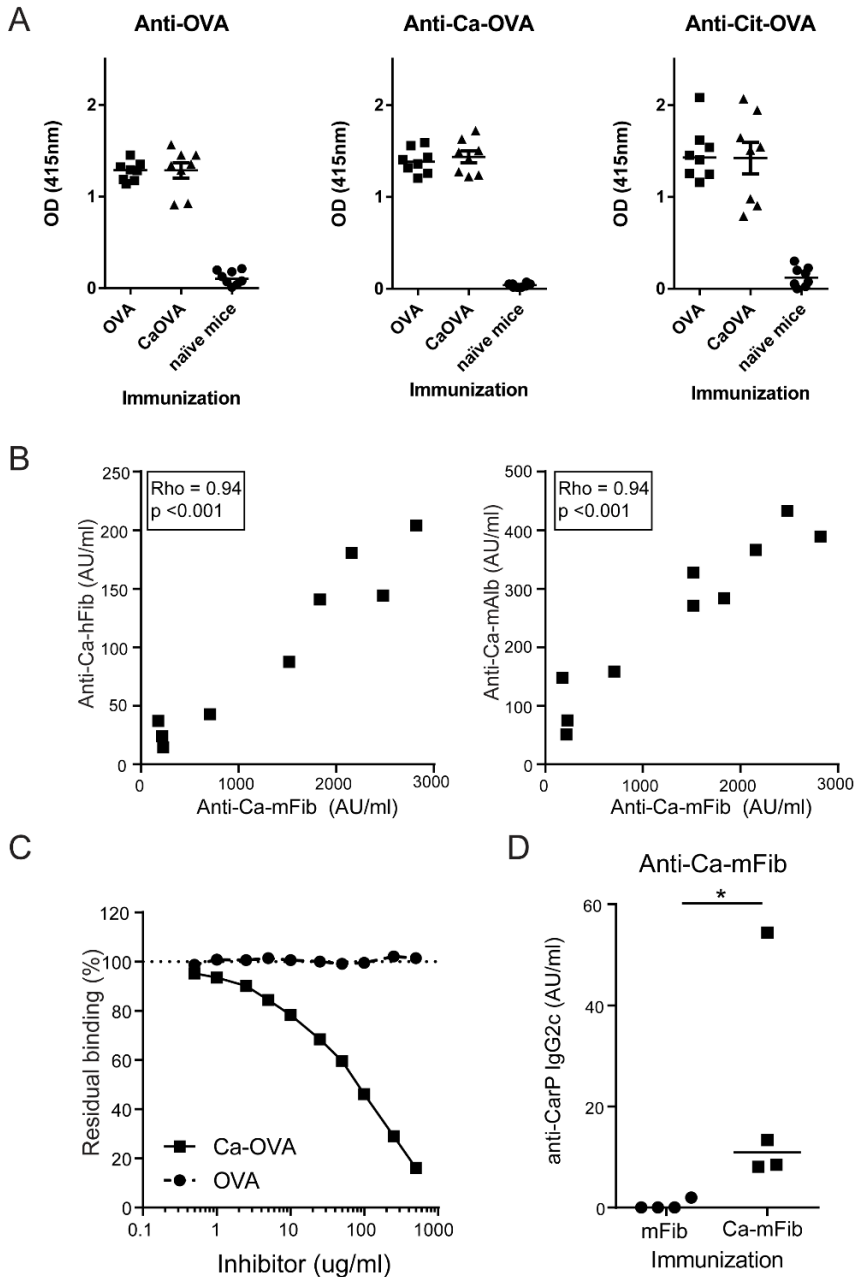
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Figure 8. Immunization with carbamylated proteins induces anti-CarP antibodies. (A) Sera from mice immunized with carbamylated ovalbumin (Ca-OVA) (squares, n=8) or non-modified OVA (triangles, n=8) and sera from non-immunized mice were tested for antibody 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.
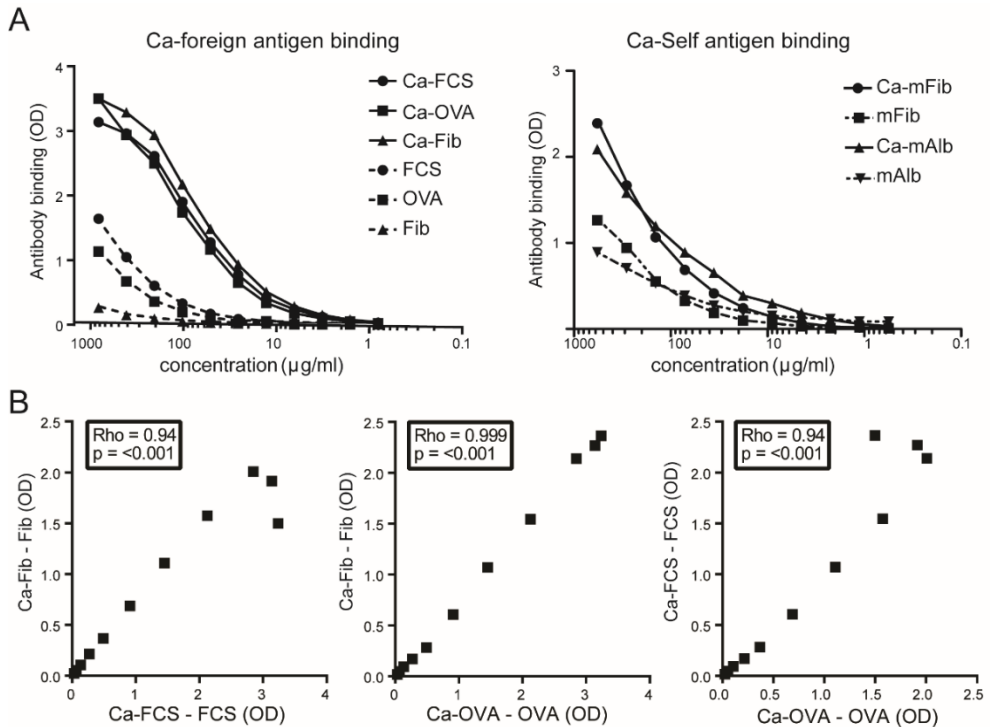
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Figure 9. Monoclonal antibodies are highly specific and cross-reactive towards carbamylated foreign and self-antigens. (A) Binding of a murine anti-CarP monoclonal antibody towards carbamylated foreign proteins; Ca-FCS, Ca-Ovalbumin (Ca-OVA), Ca-human 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

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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).
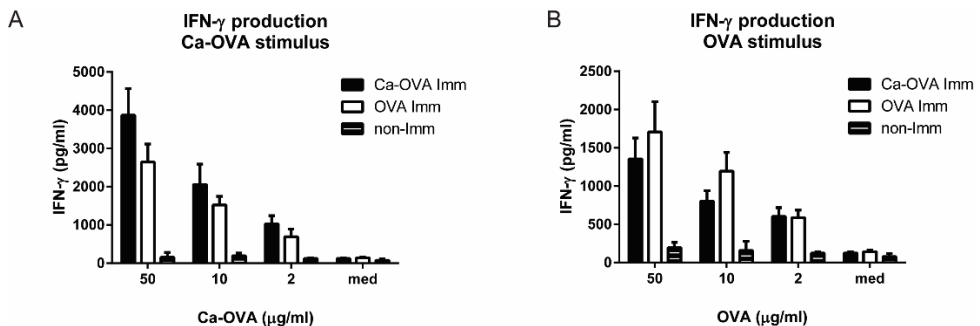

Figure 10. Interferon-γ production by bulk T-cell cultures after stimulation with ovalbumin as foreign antigen. Mice were immunized with carbamylated ovalbumin (Ca-OVA, n=9) (black bars) or non-modified ovalbumin (OVA, n=9) (white bars) in CFA and boosted with the same antigen in IFA. Spleens cells of 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.

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Chapter 3