Unravelling the anti-carbamylated protein antibody response in rheumatoid arthritis

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Chapter 11

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

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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.

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.

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.

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

Introduction

Autoimmunity in Rheumatoid arthritis (RA) patients is characterized by a spectrum of anti-modified protein antibodies (AMPA) directed against post-translationally modified (PTM) proteins. The best-known AMPA in RA are autoantibodies directed against citrullinated proteins. Anti-citrullinated protein antibodies (ACPA) target proteins that have undergone a post-translational modification of arginine into citrulline by an enzymatic process mediated by peptidylarginine deiminases (PAD)[1,2]. The identification of ACPA as specific serological marker have had a major impact on the understanding of RA and disease prognosis as their presence predicts a more destructive disease process[3-7]. Much less is known about the occurrence and aetiology of other AMPA responses in RA such as autoantibodies directed to malondialdehyde-acetaldehyde (MAA) adducts, acetylated antigens, and carbamylated proteins[8-11]. Anti-carbamylated protein (anti-CarP) autoantibodies recognize carbamylated proteins containing a homocitrulline, a PTM structurally similar to citrulline[8,12].

Like ACPA and rheumatoid factor, also anti-CarP antibodies can be detected in serum many years before RA manifestation [13-18] and similar to these autoantibodies, the presence of anti-CarP antibodies is predictive for 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]. Following activation by PTM proteins, AMPA-producing B-cells are thought to undergo the process of class switching and somatic hypermutation. 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 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. All 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 1 M potassium cyanate (Sigma-Aldrich) during 12 hours at 37 °C, followed by dialysis. Fibrinogen, mouse (Cell Sciences) and human (Sigma-Aldrich), was incubated with 0.5 M potassium cyanate during 7 days.

For citrullination; 10 mg FCS or 2 mg fibrinogen in 1 ml containing 0.1 M Tris-HCl pH 7.6 and 0.15 M $CaCl_2$ 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.

Antibody detection and inhibition assays

Human anti-CarP antibodies were detected as described previously[8]. Briefly, 10µg/ml of each antigen (carbamylated and non-modified) was coated on plates and after blocking with PBS-1%BSA, serum samples (50 times diluted) were incubated overnight at 4°C. Antibody binding was detected with HRP-conjugated rabbit-anti-human IgG (DAKO, P0214). The cut-off for positivity was set as the mean plus two times standard deviation of healthy controls.
For inhibition assays, sera were incubated with 0 or 0.2 mg/ml carbamylated or non-modified versions of one from five antigens before addition of serum samples to the ELISA plate. Mixtures were pre-incubated for 1 hour at room temperature.
Mouse anti-CarP antibodies were detected as described previously[23]. Serial dilutions of a pooled serum from mice with CIA were used as a standard.

Mass Spectrometric-analysis of joint tissue

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. Sample preparation for mass spectrometric-analysis and peptide identification is described in detail in the supplementary text.

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 week old).
For alum immunizations mice received two i.p. injections containing a 1:1 mixture of antigen (100 µg) and alhydrogel (Invivogen). Complete Freund's adjuvant (CFA) immunizations were given at the tail base, 100 µg antigen in CFA (Difco). Three weeks later a subcutaneous boost was given, 100 µg antigen in incomplete Freund's adjuvant (IFA; Sigma-Aldrich). *For* For immunizations in the absence of adjuvant, mice received two injections with 100µg antigen diluted in PBS.

Production of monoclonal antibodies

Generation of anti-CarP monoclonal antibodies, variable region cloning and antibody production is described in detail in the supplementary text.

Statistics

Prism 7 (GraphPad) or IBM SPSS Statistics 23 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 supplementary Figure 1A, 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 supplementary Figure S1B). 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.

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 (supplementary Figure S2A-B). Inhibition assays with the exact same protein used as both coating antigen and inhibitor was used as a 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 (S2C). Next ten serum samples both reactive to Ca-MBP and Ca-ProT were selected and used for subsequent inhibition experiments. Binding of anti-Ca-MBP antibodies could be inhibited by incubation with Ca-ProT (Figure 2A and SD2), unlike incubation with unmodified ProT or control.

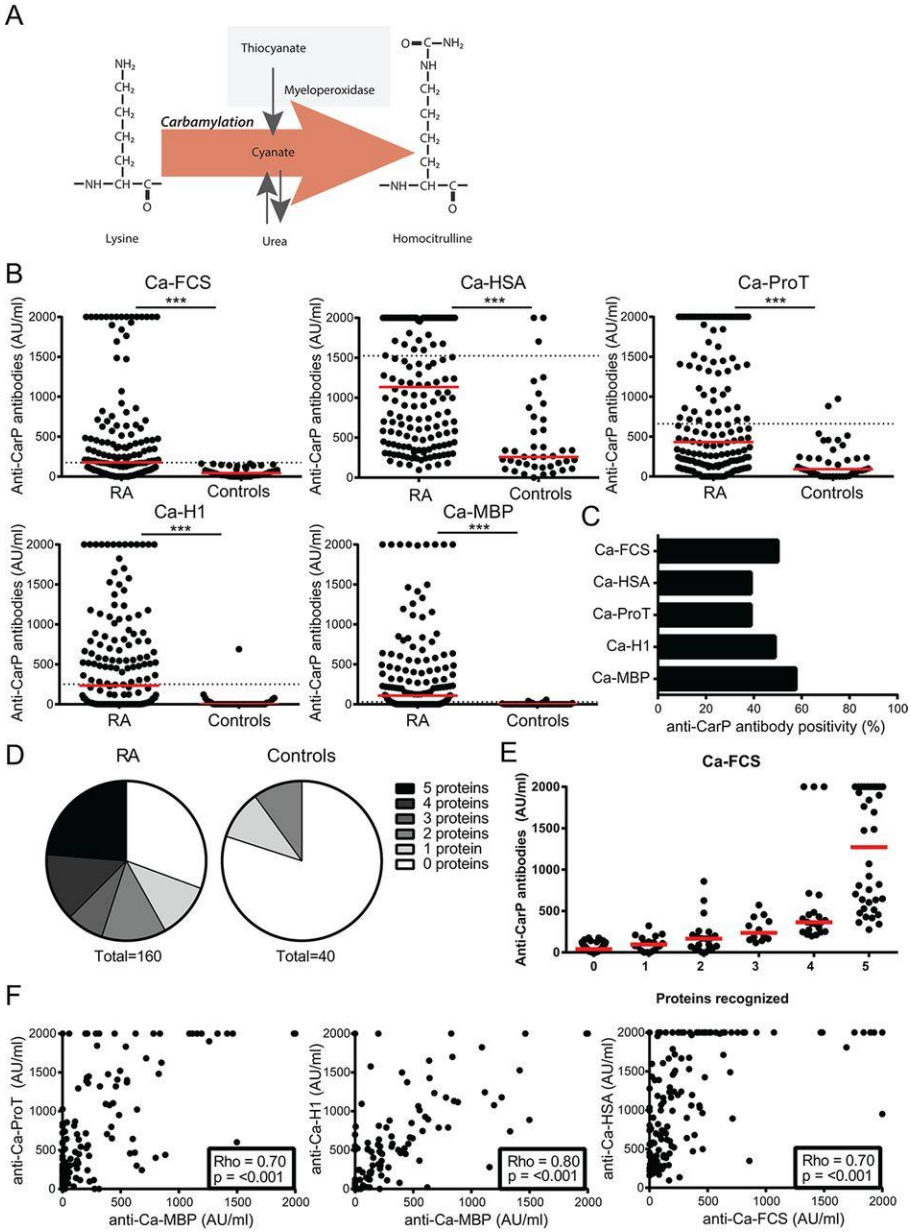

Figure 1 - Characterisation of anti-CarP antibody reactivities in patients with RA. (A) During carbamylation, a lysine residue is converted into a homocitrulline residue through a chemical reaction with cyanate. Levels of cyanate are in equilibrium with urea and can be increased, for example, during 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 patients with RA 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 patients with RA. (D) Fractions of patients that display antibody reactivity towards multiple carbamylated antigens are shown for patients with RA and controls. (E) Correlation between the amount of antigens recognised 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 millilitre; Ca, carbamylated; FCS, fetal calf serum; H1, H1 histones; HSA, human serum albumin; MBP, myelin basic protein; ProT, prothrombin; RA, rheumatoid arthritis.

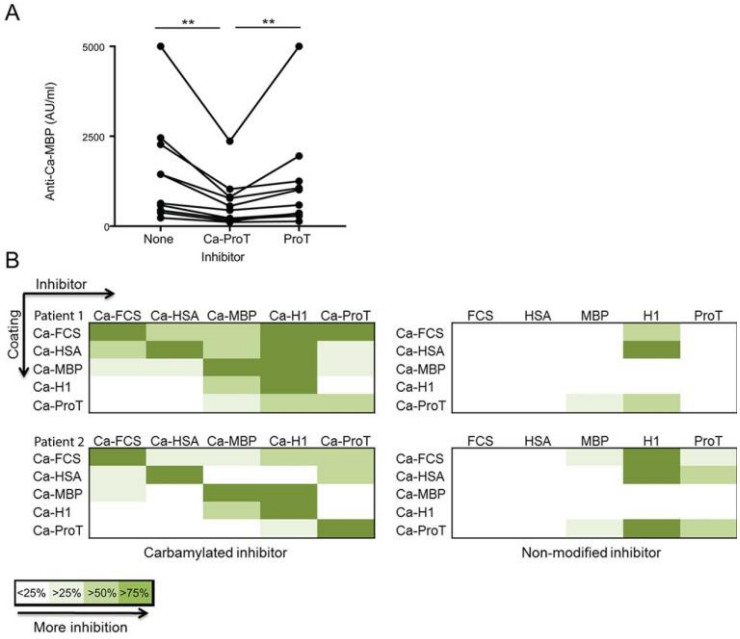

Figure 2 - Anti-CarP antibodies are highly cross-reactive. (A) Ten serum samples containing both anti-Ca-MBP and anti-Ca-ProT antibodies were used for inhibition experiments. Anti-Ca-MBP antibody binding was measured with or without the presence of Ca-ProT (0.2 mg/ml) or ProT (0.2 mg/ml) as inhibitor. 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 colour indicates an inhibition of more than 75%, followed by an inhibition of more than 50%, and the light colour indicates an inhibition of more than 25%. The white blocks indicate that the inhibition is less than 25%. anti-CarP, anti-carbamylated protein; Ca, carbamylated; FCS, fetal calf serum; H1, H1 histones; HSA, human serum albumin; MBP, myelin basic protein; ProT, prothrombin.

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 2B). 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. Together, 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.

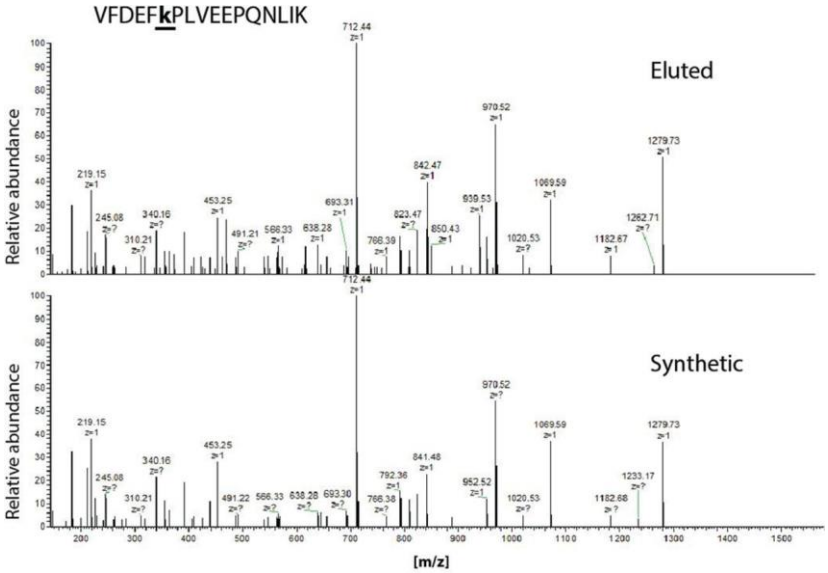

Figure 3 - Identification of carbamylated albumin in RA synovial tissue. MS2 spectrum from eluted VFDEF k PLVEEPQNLIK peptide (upper panel) derived from carbamylated albumin identified in RA synovial tissue. The synthetic VFDEF k PLVEEPQNLIK peptide (lower panel) was submitted to MS2 on the same instrument. The bold, underlined non-capital k indicates the position of homocitrulline residue. MS2, tandem mass spectrometry; RA, rheumatoid arthritis.

Carbamylated human albumin is 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 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, 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 3. 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 supplementary Figure S3A-C. Other verified proteins in which carbamylation was detected in RA patients include common proteins, such as several collagens, fibronectin, fibromodulin, albumin, and more unknown proteins including Sushi-repeat containing protein SRPX2. Although, the extent of carbamylation was not quantified, these results indicate that a carbamylated self-protein, human albumin, is present locally in the synovial compartment of RA patients.

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 4A and supplementary Figure 4A, 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 4B). 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 serum from Ca-OVA immunized mice contained antibodies recognizing carbamylated mouse Albumin (mAlb) and fibrinogen (mFib). As depicted in Figure 4C, also Ca-mAlb or 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 4D-E and supplementary Figure S4B-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 4F). In contrast, ACPA-containing sera from RA patients do recognize these citrullinated antigens.

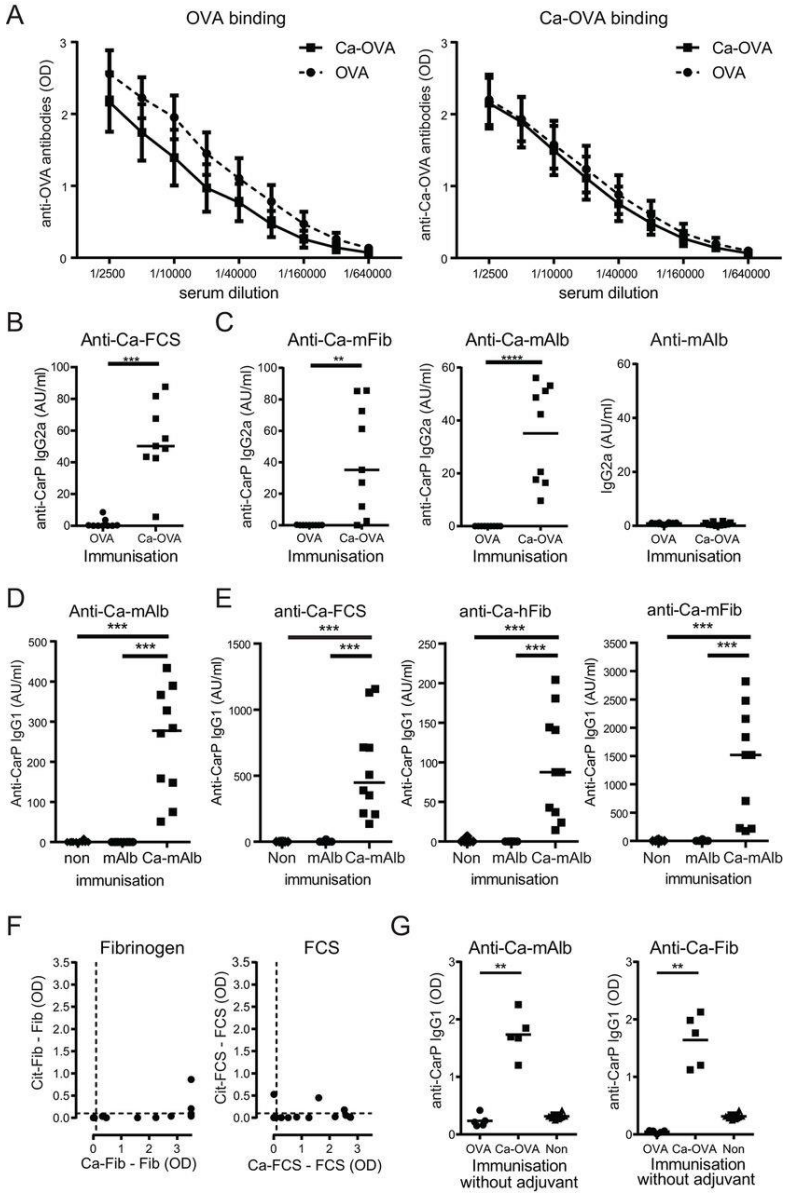

Figure 4 - Carbamylated foreign proteins can induce cross-reactive anti-CarP antibodies. (A) Mice were immunised with foreign antigens OVA or Ca-OVA. Sera from immunised mice, OVA (circles) and Ca-OVA (squares), were analysed for binding towards OVA (left panel) and anti-Ca-OVA (right panel) by ELISA (n=10). (B) Antibody reactivity towards Ca-FCS from mice immunised with OVA (circles) and Ca-OVA (squares) was determined by ELISA. Representative data from three 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 immunised mice, OVA (circles) and Ca-OVA (squares). Representative data from three experiments are shown (n= 10, Mann-Whitney U test, ***p<0.001). (D) Mice were immunised with a carbamylated self-antigen, mouse albumin (Ca-mAlb) or native albumin (mAlb) in aluminium hydroxide. Sera from immunised mice (Ca-mAlb) (depicted as squares), mAlb (depicted as circles) and non-immunised mice (depicted as triangles) were analysed for reactivity towards Ca-mAlb. Representative data from three 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 immunised mice were analysed 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-immunised mice (depicted as triangles) were analysed by ELISA. Representative data from three 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-immunised 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 immunised with foreign antigens OVA or Ca-OVA in the absence of adjuvant. Sera from immunised mice OVA (circles), Ca-OVA (squares) and non-immunised mice (triangles) were analysed for binding towards carbamylated self-proteins, Ca-mAlb (left panel) and Ca-mFib (right panel) (n= 5, Mann-Whitney U test, **p<0.01). anti-CarP, anti-carbamylated protein; Ca, carbamylated; FCS, fetal calf serum; IgG, immunoglobulin G; OD, optical density; OVA, ovalbumin.To examine whether carbamylated foreign proteins can break tolerance in absence of adjuvants, we next immunized mice with Ca-OVA in PBS. Interestingly, significant antibody responses against carbamylated self-proteins were induced (Figure 4G), showing that immunization with carbamylated foreign proteins in absence of adjuvant also results in a cross-reactive B-cell response against modified self-proteins.

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 5A, this monoclonal antibody binds both carbamylated foreign- and self-proteins. We observe a significant correlation between the monoclonal antibody binding to Ca-FCS and Ca-OVA and between Ca-Fib and Ca-FCS (Figure 5B) confirming its cross-reactive nature. Thus, as observed for polyclonal anti-CarP antibodies from mice immunized with a foreign antigen, strong cross-reactivity is observed towards different carbamylated foreign and self-proteins at the monoclonal antibody level, confirming that self-reactive AMPAs can be induced by exposure to foreign PTM proteins.

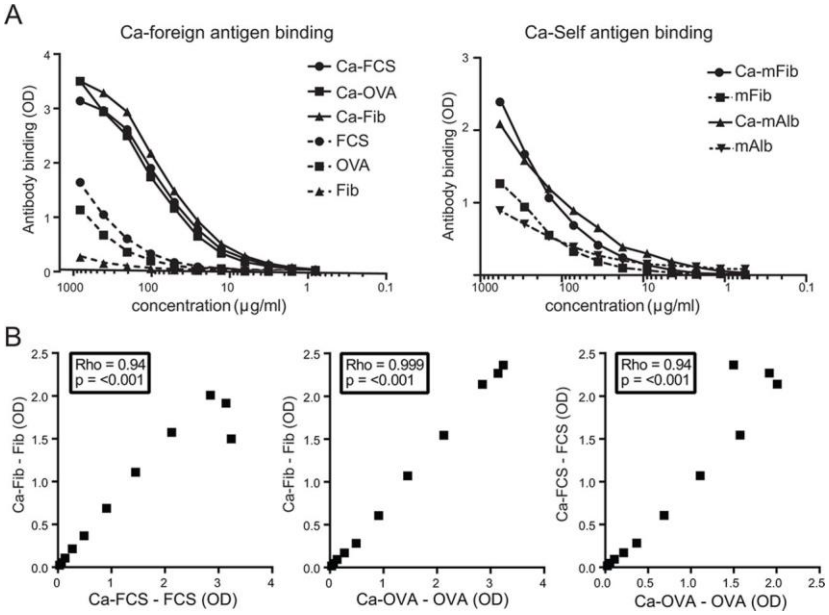

Figure 5 - Monoclonal antibodies are highly specific and cross-reactive towards carbamylated foreign and self-antigens. (A) Binding of a murine anti-CarP monoclonal antibody towards carbamylated foreign proteins; Ca-FCS, Ca-OVA, Ca-Fib (human fibrinogen) and non-modified counterparts were tested using ELISA (left panel). Reactivity of the anti-CarP monoclonal antibody towards carbamylated self-proteins; Ca-mFib (mouse fibrinogen), Ca-mAlb (mouse albumin) and non-modified counterparts were 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 with 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). anti-CarP, anti-carbamylated protein; Ca, carbamylated; FCS, fetal calf serum; OD, optical density; OVA, ovalbumin.

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.

Although cross-reactive AMPA responses have been described [9,10,26], it has not been demonstrated that AMPA-producing B-cells recognizing a particular modified self-protein can be induced by other -unrelated- modified proteins. Previous animal studies showed that immunogenicity of proteins is enhanced upon citrullination, but did not show whether immunization with a modified (structurally unrelated) foreign protein leads to the induction of a cross-reactive AMPA-response against self[27-29]. Clearly, human studies demonstrating this principle are challenging as the autoantibody-inciting events are unknown and difficult to control[30].
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[9,10,26]. For example, previous human studies have shown that also ACPA exhibit cross-reactive properties towards different citrullinated self- and foreign antigens[26,31-35]. 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 [36-39], it is highly conceivable that similar principles as identified for anti-CarP-antibody responses apply to other classes of AMPA as well.

Although it is unknown how autoantibodies against PTM proteins are generated in humans, it is often speculated that an autoreactive T-cell response recognizing such self-proteins is crucial for their appearance. Clearly, our results are not incompatible with this notion and do not indicate that such T-cell help would not contribute the induction of B-cell mediated autoimmunity against PTM proteins. However, our results provide first evidence that also T-cells recognizing “conventional” foreign antigens could be involved in the induction of AMPA-producing B-cell responses that recognize modified self-proteins. So far it is unclear to what extent T-cell tolerance is lost in RA as identification of PTM epitopes recognized by autoreactive T-cells has been proven difficult. Although T-cell responses against PTM self-proteins have been described [40-42], frequencies of citrulline specific T-cells are only about 1 in 100,000 CD4 cells compared to 1:10,000 CD4 cells for tetanus toxoid specific T-cells[42,43].

Data from animal studies suggest that PTM proteins can generate antigen-specific T-cell responses in mice [28], [19]. However, 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 (supplementary Figure S5), 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 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), the release of myeloid peroxidase leading to enhanced carbamylation or the presence of bacterial-derived acetylated proteins[1,12,44]. 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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