# Tolerance and immune regulation in rheumatoid arthritis

Dekkers, J.S.

# Chapter 4

## Carbamylated autoantigens facilitate a breach in T cell tolerance

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**ABSTRACT**

**Objectives**

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

**Methods**

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

**Results**

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

**Conclusions**

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

**Introduction**

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

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

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

## Methods

### Carbamylation of mouse Albumin

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

### Mice and immunizations

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

### T cell assays

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

### Protein digestion and mass spectrometry

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

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

**Statistical analysis**

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

**Results**

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

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

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

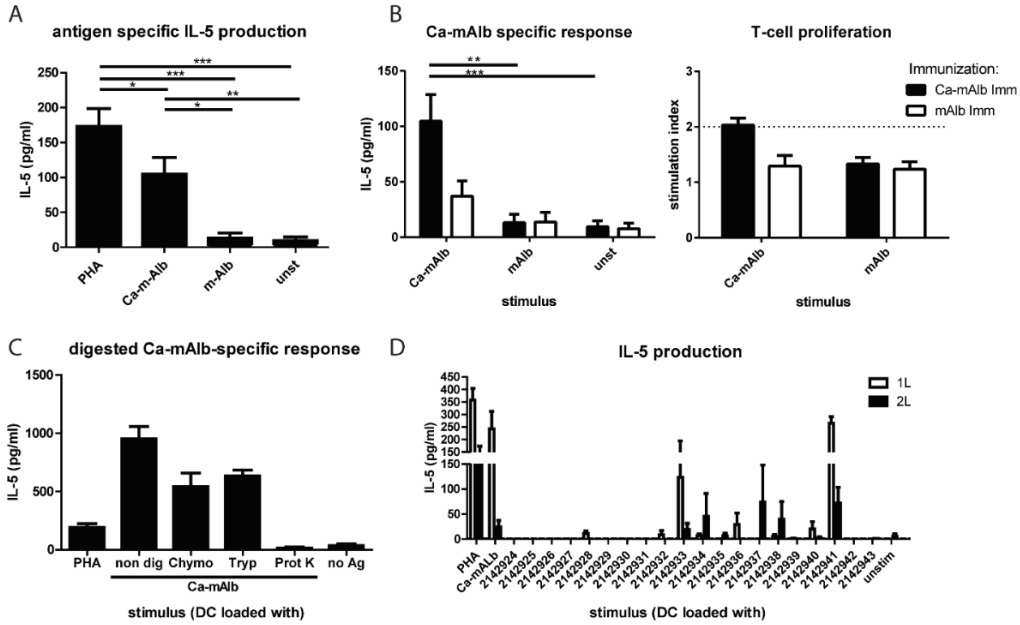


**Figure 1. T cell activation and cytokine production after stimulation with carbamylated mouse Albumin.** (A) Mice were immunised with carbamylated mouse albumin (Ca-mAlb) or native mouse albumin (mAlb) in aluminium hydroxide and boosted with the same antigen mixture. Spleen cells were *in vitro* stimulated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

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

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

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

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

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

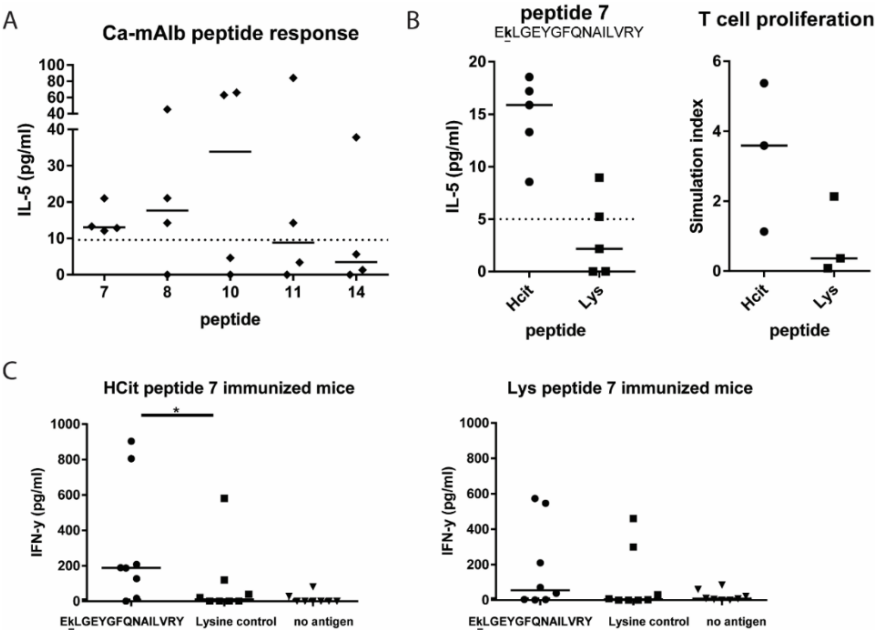


**Figure 2. Homocitrulline specific T cell responses after stimulation with carbamylated peptide 7 (A)** Mice were immunised with carbamylated mouse albumin (Ca-mAlb) in aluminium hydroxide and boosted with the same antigen mixture. Spleen cells were *in vitro* stimulated with D1 cells pulsed with different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

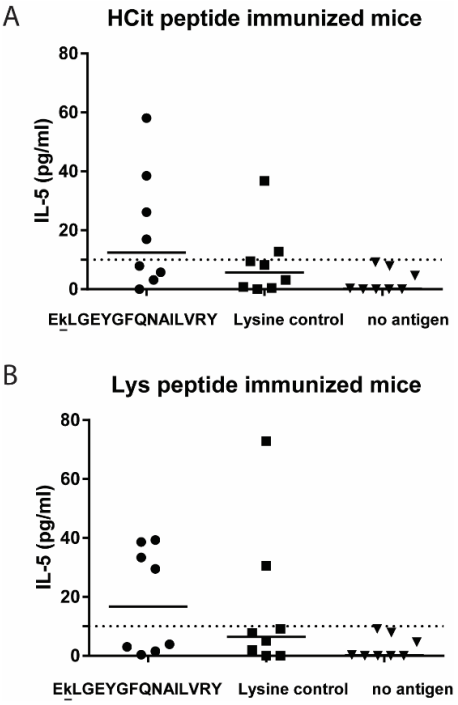


**Figure 3. Low levels of IL-5 production after stimulation with carbamylated peptide 7** Mice were immunised with a carbamylated **(A)** or non-modified version of peptide 7 **(B)** using aluminum hydroxide. Spleen cells were *in vitro* stimulated with D1 cells pulsed with carbamylated peptide 7, non-modified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

**Discussion**

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

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

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

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

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