Immunity against post-translationally modified proteins in autoimmune diseases

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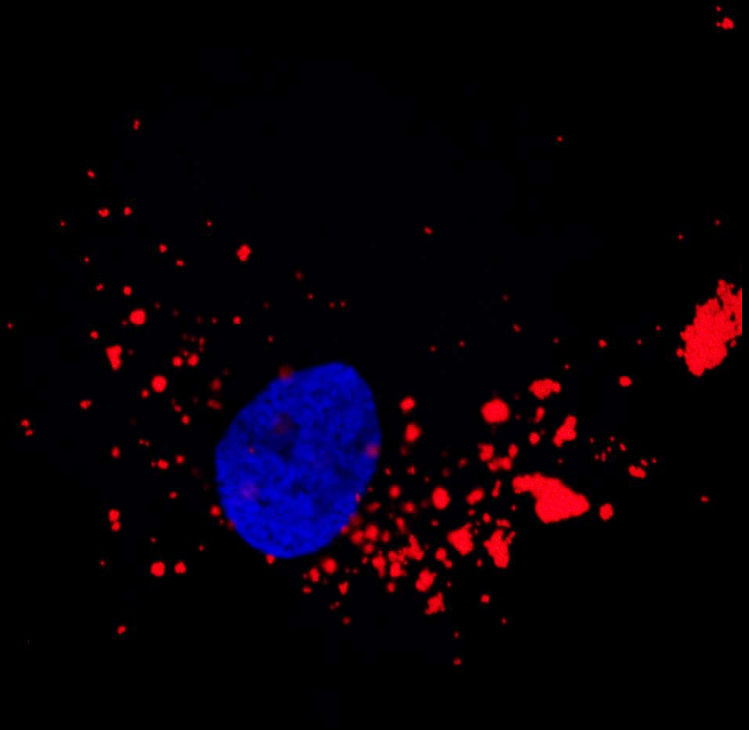

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General discussion

Autoimmune diseases are a diverse group of conditions in which the immune system mistakenly damages healthy cells, tissues, or organs. Many autoimmune diseases are therefore characterized by the presence of autoantibodies. Some of these antibodies are disease specific and some are observed in several conditions. One particular example are antibodies that target post-translational modifications (PTMs), which are the main focus of this thesis. Antibodies targeting PTMs are observed in several major autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Antibodies targeting the modification citrullination (ACPA) in RA are disease specific and are therefore included in the 2010 rheumatoid arthritis classification criteria (1). Other anti-PTM antibodies, such as antibodies targeting carbamylated protein (anti-CarP) are associated with in joint damage in RA (2), but also in arthralgia and SLE joint damage occurs (3, 4). In these patients, anti-CarP antibodies may serve as a promising marker to predict joint damage. In this thesis we focused on anti-PTM antibodies directed against six different PTMs namely, nitration (Nt), citrullination (Cit), carbamylation (Ca), acetylation (Ac), malondialdehyde acetaldehyde adduct (MAA) and advanced glycation end-product (AGE). We selected these 6 PTMs as they are relatively easy to produce, were either structurally different, occurred on the same or on different amino acid residues and because these 6 PTMs are differentially associated with several types of disease. The relevance of these six PTMs was investigated by analyzing anti-PTM antibody reactivities and performing association studies in patients with RA (**Chapter 2**), SLE (**Chapter 3**) and autoimmune liver disease (AILD) (**Chapter 4**). In an attempt to understand how tolerance is broken towards PTM proteins we studied the interaction between complement and PTM-modified proteins (**Chapter 5**). With these studies we provide insight into the presence of anti-PTM antibodies and mechanisms by which PTM and anti-PTM antibodies are contributing to (chronic) inflammation of autoimmune diseases.

The clinical relevance of anti-PTM antibodies in RA

Autoantibodies targeting PTMs are frequently present in patients with RA. More specifically, anti-PTM antibodies that target citrullinated (ACPA) and carbamylated (anti-CarP) proteins are widely used as diagnostic and prognostic markers (2, 5). However, despite the high diagnostic value of ACPA and rheumatoid factor (RF) a substantial group of RA patients is reported to be seronegative (6). Therefore, there is a need for novel serological markers to further improve early diagnosis of RA also in such seronegative patients. Many research groups are focusing on new targets of autoantibody reactivity. It is becoming apparent that next to ACPA and anti-CarP also other PTMs are targeted in these patients (7-10). In this thesis we investigated the six different anti-PTM antibody reactivities (anti-Nt, -Cit, -Ca, -Ac, -MAA and -AGE).. These anti-PTM antibody reactivities were assessed in a well-established cohort of RA patients, namely the Leiden Early

Arthritic Cohort. In **Chapter 2** we focus on anti-PTM antibodies targeting MAA- and AGE-modified proteins and their clinical relevance in RA (11). RA patients were significantly more frequently positive for the presence of anti-MAA and anti-AGE antibodies. On top of that, presence of anti-MAA and anti-AGE identified a subgroup of RA patients that was seronegative for ACPA, anti-CarP and rheumatoid factor (RF). These findings underpin that patients with RA display a heterogeneous array of autoantibodies, each associated with more severe disease and therefore possibly contributing to disease pathogenesis. Noteworthy is that a subgroup of RA patients remains seronegative. Serological markers for these seronegative RA patients may be identified in the future, further identifying subgroups. However, it is possible that a subgroup of RA patients remains seronegative, as the association with HLA is present especially in the currently seropositive patients and not with the seronegative patients. Recently, the immune response to PTM-modified proteins in RA was reviewed in detail (12). Here they focused on cross-reactive capabilities of anti-PTM antibodies and suggest that citrullinated proteins are probably the dominant antigen in the anti-PTM response in patients with RA (12). Intriguingly, PTM-directed B cell responses in RA does not seem to transition into a resting state, but remain consistently activated (13). When aiming for sustained clinical remission, this may imply that disease-associated immune response most likely remain active despite treatment-induced clinical remission. This provides an immunological rationale for the observed disease flares on drug tapering withdrawal.

The clinical relevance of anti-PTM antibodies in other autoimmune diseases

Next to RA, many other autoimmune diseases display a variety of autoantibodies targeting (modified) self-proteins. Additionally, PTMs and antibodies targeting PTMs have been identified in several non-autoimmune diseases such as age-related macular degeneration, cardiovascular disease and diabetes type 2 (14-16). It is therefore likely that anti-PTM antibodies, or combinations of several anti-PTM antibodies, are present in many autoimmune diseases. Next to RA, we therefore investigated the set of six different anti-PTM antibody reactivities in cohorts with SLE patients and patients with AILD. In **Chapter 3**, we had the unique opportunity to investigate SLE patients with neuropsychiatric symptoms since the Leiden University Medical Center is a tertiary referral center for these patients (17). No serological markers are currently available to identify patients with neuropsychiatric SLE (NPSLE), therefore such research was warranted. We observed that patients with SLE more frequently had anti-PTM antibodies targeting CarP-, AGE- and MAA-modified proteins. On top of that, anti-CarP and anti-MAA were more frequently present in patients with NPSLE. Additionally, anti-PTM antibodies negatively correlated with brain volumes, an objective marker of central nervous system

involvement. In **Chapter 4**, patients with AILD were assessed for the presence of the six anti-PTM antibodies (18). Anti-PTM antibodies (anti-CarP, anti-Ac, anti-MAA, anti-AGE) were more often detected in sera from AILD patients compared to healthy controls. The presence of anti-MAA, anti-AGE and anti-CarP antibodies correlated with the presence of autoimmune hepatitis (AIH) within this cohort. On top of that, in AIH, harboring at least three anti-PTM antibody responses is positively associated with complete biochemical response. In a reply on a commentary written on **Chapter 4** (included in Chapter 4) we further explained the methods used to measure anti-PTM antibodies. The experimental set-up is based on the anti-CarP assay (2, 19). This method includes, and corrects for, antibody responses towards the backbone of non-modified FCS we are therefore certain to measure specifically anti-PTM antibodies in serum samples.

Overall, we have shown that measuring anti-PTM antibodies in patients with autoimmune diseases may have diagnostic and prognostic value. In addition, the presence of anti-PTM antibodies may provide information on the response to therapy. Further research is needed to establish sensitivity and specificity. We observed that similar PTMs were targeted by anti-PTM antibodies throughout different autoimmune diseases. Noteworthy is that, many biomarkers have already been suggested in several autoimmune diseases, but for many of these suggested biomarkers the role is yet to be determined and clinical value limited (20, 21). It is therefore unlikely that within the whole population one of these anti-PTM antibodies on its own distinguish (at risk) patients from healthy individuals. We, however, have observed that within a specific cohort anti-PTM antibodies, or combinations of anti-PTM antibodies, may determine a relevant subgroup of patients. Many autoimmune diseases namely consist of a heterogenous group of patients. Determination of subgroups may provide better insight in disease progression or treatment response.

During our studies we observed that anti-AGE and anti-MAA correlate with inflammatory markers. Previous studies have shown that the PTM AGE binds to the receptor RAGE amplifying immune and inflammatory responses (22). Both AGE- and MAA-modified proteins have previously shown to be immunogenic (23, 24). The role of MAA-modified proteins as mediators of inflammation in cardiovascular diseases have been reviewed in detail elsewhere (25). These studies, together with our observations, suggest that PTMs themselves may be a result of inflammation and oxidative stress but also play a role in perpetuation of inflammation. This vicious circle, may under the ''right'' circumstances, lead to chronic inflammation and autoimmunity. In addition, **Chapter 5** we show that the PTMs Ca, Ac, MAA and AGE have the capacity to trigger complement activation. Collectively indicating that both the PTMs and the anti-PTM antibodies have the capacity to stimulate (chronic) inflammation.

The observation that next to RA also other diseases are characterized by anti-PTM antibodies, provides important insight into the pathophysiological processes that take place in these diseases. However, it does not necessarily indicate that the detection of anti-PTM antibodies in RA for diagnostic or prognostic purposes would be less valuable. The assays set up to determine ACPA, often the CCP2, CCP3 or CCP4 based assays are still very relevant. Also the assay to detect anti-CarP antibodies, can still be employed for their diagnostic and prognostic purposes in RA. These assays have been established employing sets of relevant disease controls and cut-offs for positivity have been determined for optimal use in the clinical setting. However, the data do importantly indicate that in several autoimmune conditions, known to involve autoantibodies, anti-PTM antibodies can readily be detected in a large part of the patients. Importantly, the anti-PTM antibodies are not present in all the patients as some patients with RA, SLE or AIH harbor none of the anti-PTM antibodies investigated. Combining the data from the three cohorts, as well as studying the cohorts separately, we observed that there are several patterns of positivity. Patients can be positive for all six, or for none, but all other combinations were observed as well. The highest percentage positivity was observed for anti-MAA in both SLE and AILD. In RA, anti-MAA was found in 46,7% of all patients and was therefore the second most common anti-PTM antibody in RA, next to anti-Cit (49,3%). When all data of all autoimmune diseases were pooled, we observed that antibodies targeting Cit, CarP, Ac, MAA and AGE mostly co-occurred in autoimmune disease followed by the combination of anti-CarP and anti-MAA antibodies. Taken together, our observations indicate that despite the fact that all these patients are characterized by major inflammatory events, that only a part of the patients will actually break tolerance and produce such autoantibodies.

Several reports have looked into the cross-reactive nature of anti-PTM antibodies and while there is certainly a degree of cross-reactivity present for some of the anti-PTM antibodies it is also obvious that PTM specific, non-cross reactive antibodies are present. For example evidenced by patients that are single positive for one anti-PTM. The degree of cross-reactivity may appear different when analyzing anti-PTM antibodies on PTM containing CCP peptides as compared to PTM containing proteins. Work that is currently in progress at the Department of Rheumatology is highlighting that apparent cross-reactivity is generally higher in CCP based assays as compared to protein based assays. Conceivably, this is because of the structure of the CCP peptide backbone that was optimized to capture as many RA patients as possible compared to controls. Future work, based on human monoclonal anti-PTM antibodies will have to disclose the cross-reactive nature also including the full set of six PTM reactivities.

The possible role of complement in the induction of anti-PTM antibodies

PTMs and antibodies targeting these PTMs are the main subjects of this thesis. It has become apparent that PTMs and anti-PTMs are frequently present, in both health and disease (26). In **Chapter 5** we aimed to shed light on how the human body deals with PTM-modified proteins and identified triggers that may lead to autoimmunity (Figure 1) (27). Specifically we asked the question if serum proteins could bind to PTMs to modify their clearance and immune stimulating capacity. We observed that, from human serum, complement components bind directly to certain PTM-modified proteins (Ca, Ac, MAA and AGE). Notably, seven out of the top ten hits observed using mass spectrometry were complement components, suggesting an important role of complement binding to PTM-modified proteins. The complement system is perceived as a central constituent of innate immunity, defending the host against pathogens, coordinating various events during inflammation, and bridging innate and adaptive immune responses.

We have established that complement not only binds directly to PTMs, but also that complement binding to PTM-modified proteins stimulates the uptake of PTM-modified proteins by macrophages. Next to macrophages, leukocytes bind complement opsonized PTM-modified proteins more compared to non-opsonized PTM-modified proteins.

Interestingly, most people have PTM-modified proteins at several locations in their body and have a functional complement system, but not all people develop an anti-PTM antibody response. We therefore set out to analyze genetic variants of complement single nucleotide polymorphisms (SNPs), associated with a more active complement profile, so called complotype, and correlated these with anti-PTM reactivities. We observed that patients with a more complement activating profile, were associated with anti-PTM antibodies. We observed that minor allele factor H SNPs, previously shown to increase complement activity, associated with presence of anti-Ac, -MAA and -AGE. The minor allele of rs2230199 (C3) associated with anti-Ca. Interestingly, the minor allele of C1qA rs292001 associated with anti-Cit, contributing to the findings that C1q SNPs are described in the context of RA. However, the PTM Cit itself did not show complement activating properties, suggesting a different mechanism of antibody induction for this particular PTM.

We hypothesized that the complement system will be activated on PTM-modified proteins regardless of the complotype and that C1q or ficolins will bind to specific PTM-modified proteins. Following binding of C1q or ficolins the complement cascade will be further activated resulting in amongst others deposition of C3 fragments (C3b, C3d, iC3b) serving as opsonins for cells to be detected through complement receptors. The complotype will

determine how strong this reaction will be. Next to opsonization, complement activation will also trigger the release of the anaphylatoxins C3a and C5a stimulating immune cell influx and immune cell activation. Ultimately, complement activation can result in the formation of the membrane attack complex, leading to cellular activation or even cellular death by apoptosis or direct lysis. Therefore we hypothesized that, depending on the complotype, people will clear the PTM-modified proteins, or alternatively upon highly active complement sustain inflammation and might mount an antibody response. Nonetheless, we think that it is an interplay of genetic and environmental factors that will eventually lead to disease in certain people.

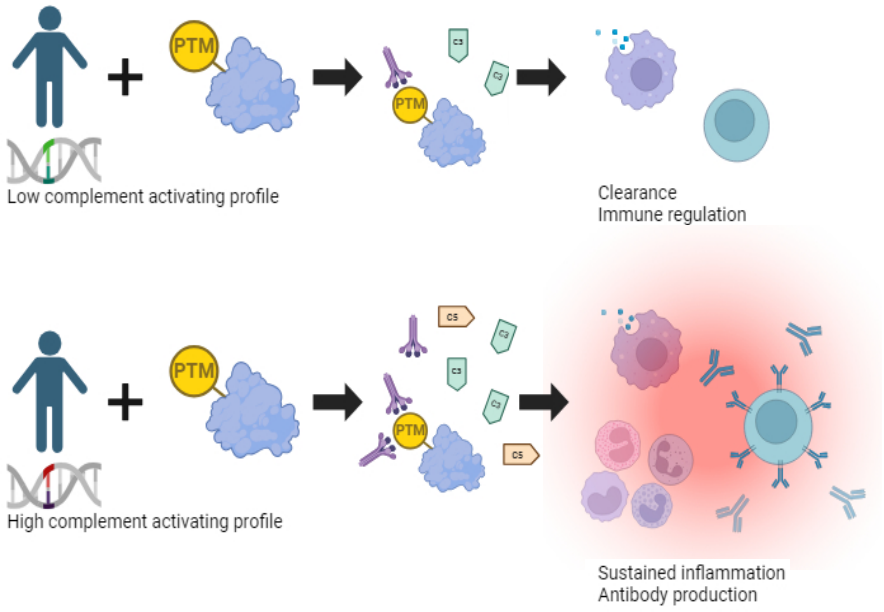

Figure 1: Key hypothesis. The so-called complotype of individuals results in a low (upper part) or high (lower part) complement activating profile. Depending on the complotype, complement opsonization of PTM-modified proteins could lead to clearance and immune regulation or (sustained) inflammation and autoantibody production, respectively.

To study the impact of complement opsonization of PTM-modified proteins on the triggering and activation of anti-PTM specific B-cells it would have been great to stimulate patient derived anti-PTM reactive B-cells with PTM-modified protein or with complement opsonized PTM-modified protein. However, only a few B-cells in these patients will be PTM specific. Since the amount of PTM-specific B-cells is limited, no experiments could be performed to investigate the induction of antibodies by our six PTM-modified proteins in the presence or absence of complement. However, several groups have studied the impact of complement

opsonization of proteins in their capacity to stimulate B-cell responses. The concept is that complement receptor 2, expressed on B-cells, binds to C3d lowering the B-cells activation threshold. Kovacs *et al.* has shown that, in mice, tolerance of B-cells is decreased when the B-cell receptor was activated non-specifically the presence of complement factor C3d, using anti-IgG/A/M F(ab')2 fragments coupled to C3d (28). In the near future this should be investigated in human in order to strengthen the hypothesis that a breach in tolerance could be induced against complement opsonized PTM-modified proteins by PTM specific B-cells.

Others have investigated anti-PTM reactivities in a different approach by assessing cross-reactivity (29, 30). They hypothesized that only one PTM may lead to the induction of several anti-PTM reactivities. In the context of RA, they have created B-cells with citrullinated protein-reactive IgG B-cell receptors. Indeed, they observed that these B-cells showed activation on stimulation with various types of PTM-antigens (Cit, Ca and Ac) (29). The B-cell model used in this study were Ramos cells. Unfortunately, the cell line Ramos lacks expression complement receptor 2. Additionally, Cit was one of the PTMs that did not show complement activation in our studies. Therefore, this model could not be used to investigate whether complement lowers the B-cell activating threshold in PTM-specific B-cells.

The same group recently published findings on acetylated gut-resident bacteria induce cross-reactive anti-modified protein antibodies in mice (31). It is postulated that gut microbiome is involved in the breach in tolerance to modified self-proteins. Additionally, the genetic-environmental interaction has been found for HLA-SE alleles and smoking (32, 33). Smoking namely leads to increased citrullination in pulmonary tissue which could be subsequently presented in HLA-SE alleles. This hypothesis was backed up by data including presence of citrullinated antigens, ACPA-positive B-cells and ACPA in bronchoalveolar lavage samples before and after onset of RA (34-36).

Combining data from these studies with our observations it is theoretically possible that, depending on environmental and genetic factors, strong immune reactions are induced against specific PTM-modified proteins. It is however important, based on the phases of disease evolution, to distinguish risk factors that contribute to the initial development of autoantibodies, from those that affect later stages such as symptom onset (12). Our findings showed that certain PTMs could directly activate complement which led to increased binding and uptake by leukocytes and macrophages. Under the ''right'' circumstances this could lead to the breach in tolerance of PTM-specific B-cells that will produce anti-PTM antibodies with cross-reactive potential targeting several different PTM-modified proteins. These anti-PTM reactivities will be detectable in patients. We would however, like to note that not all patients display similar cross-reactivity. We

namely observed that patients can be positive for multiple anti-PTM reactivities but clearly not all for the same combinations.

The findings that PTM-modified proteins activate complement directly may have important implications for the understanding of smoldering, chronic inflammation. PTM-modified proteins present in joints, kidneys or vessels may be a chronic stimulus for low level complement activation leading to a vicious circle of enhanced PTM formation, complement activation, triggering of inflammation, which in turn again leads to PTM formation.

Notably, not all PTM-modified protein induce anti-PTM antibody reactivities in all patients. Anti-Nt antibodies, namely, were only observed in a few individuals. These data suggests that anti-Nt antibodies do not play a substantial role in the autoimmune diseases investigated in this thesis. On top of that, both Nt and Cit did not activate complement suggesting a different type of mechanism that lead to the induction of anti-PTM antibodies. Besides, in this thesis all lysine modifications induced complement activation. However, we would like to point out that not every lysine modification does this similarly and to the same extend. Ca-, MAA- and AGE-modified proteins activate the classical pathway and Ac-modified proteins activate the lectin pathway. Additionally, MAA-modified proteins bind C1q already at very low concentrations exerting great complement activating potential. For future studies it is important to keep in mind that not all PTM-modified proteins react similarly and different mechanism leading to disease onset, but also at a later stage considering disease symptoms, might be involved for each PTM-antigen.

Conclusion and future perspective

At this point, we have established that anti-PTM antibodies are present in a variety of autoimmune diseases including RA (**Chapter 2**), SLE (**Chapter 3**) and AILD (**Chapter 4**). In the cohorts tested we observed that anti-PTM antibodies and combinations of anti-PTM antibodies distinguish subgroups, correlate with specific manifestations or correlate with a treatment outcome. Since many autoimmune diseases are characterized by autoantibodies, including antibodies targeting PTMs, it is challenging to pin-point specific PTMs associating with specific disease. It is more likely that certain anti-PTM antibodies or combinations of anti-PTM antibodies are present in specific subgroups and associate with disease outcome. Prospective studies comprising different autoimmune diseases could shed light on diagnostic and prognostic value of these anti-PTM antibodies.

We have established that the complement system is activated by specific PTMs (**Chapter**

5). Additionally, complement-opsonized PTM-modified proteins show an increase in cellular uptake. This data is based on plate bound assays using complement active human serum and human cells or cell lines. To further elucidate on the relevance of the complement system in the induction of anti-PTM antibodies, mouse studies should be performed. In such studies mice deficient for specific complement components should be used to identify the role of complement in the breach in tolerance. For example, mice lacking complement component C1q, important for the classical pathway, should be exposed to Ca-, AGE- or MAA-modified protein and subsequently anti-PTM reactivities measured. When antibody reactivity is compared to antibody reactivity in mice with a normal complement system, this would give insight in the contribution of complement activation to the induction of anti-PTM antibodies.

Results observed in **Chapter 5**, however, are convincing and could be a great basis for further immunotherapeutic purposes. In RA, but also in other autoimmune diseases, broad immunosuppression is current treatment and sustained clinical remission the golden standard (37-39). The possible role of complement in the induction of anti-PTM reactivity provides opportunities to modify complement activation and therefore lower the chance of autoimmunity. However, therapeutic targeting of the complement system is easier said than done (40). Suppressing complement systemically for instance is undesired as the complement system is needed to fight infection, heal injury and kill bacteria and viruses (41). Data provided in this manuscript provides ground work for new therapeutic interventions. Proteins modified with Ca, Ac, MAA or AGE activate the complement system directly and are shown to occur in several autoimmune diseases. Direct complement activation implies that complement inhibition may serve as therapeutic intervention in these patients. Using prolonged and systemic complement inhibition may be disproportional to the chronic complement activation by the PTMs, especially in light of the risk of infections during such therapy. Suppressing complement activation locally, only on PTM-modified proteins, for example using bispecific antibodies could serve this purpose very well (42). This strategy would fit in the proposed interventions aiming for sustained immunological remission leading to sustained (drug-free) remission (12). In the mentioned strategy, bispecific antibodies, are able to bind two different antigens as opposed to normal antibodies only targeting one antigen. In this way, a bispecific antibody was designed that with one arm can bind to the PTM and the other arm to endogenous complement regulator Factor H, bringing complement inhibitors into close proximity to the PTMs regulating complement activation.

Altogether, throughout this thesis, I described a role for immunity against PTMs in a variety of autoimmune diseases. Further exploration of triggers towards PTMs and anti-PTM antibody induction is warranted to establish treatment options for patients aiming for sustained immunological remission.

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