Immunity against post-translationally modified proteins in autoimmune diseases

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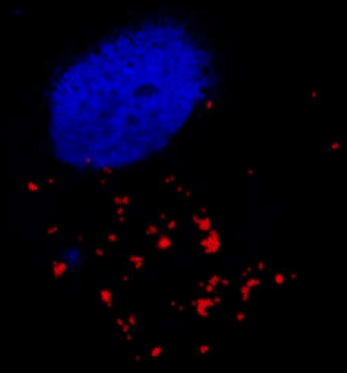

7

Appendices

English summary
Nederlandse samenvatting
List of publications
Curriculum vitae
Dankwoord

English summary

Autoimmune diseases are conditions in which the immune system mistakenly attacks its own healthy cells, tissues, or organs. Such diseases are often characterized by chronic inflammation. Conditions such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and autoimmune liver disease (AILD) exemplify the diversity and complexity of autoimmune disorders. These heterogenous diseases are characterized by the presence of a wide array of autoantibodies. Patients with RA harbor antibodies against proteins that have undergone a post-translational modification (PTM). Antibodies targeting the modification citrullination (anti-citrullinated protein antibodies; ACPA) are included in the 2010 rheumatoid arthritis classification criteria. Antibodies targeting the modification carbamylation (anti-carbamylated protein; anti-CarP) are reported to be associated with radiological progression, especially in ACPA negative individuals. Other autoimmune diseases such as SLE and AILD are hallmarked by the presence of amongst others anti-nuclear antibodies. These antibodies have shown to be pathogenic and associate with severe clinical complication in SLE and aid in the diagnosis of AILD.

For all three autoimmune diseases (RA, SLE and AILD) it is important to note that within each disease, many subgroups of patients exist. These subgroups may be characterized by specific autoantibodies and vary in speed of progression and/or display specific disease manifestations. In addition, patients in such subgroups can also respond differently to specific therapies. It is therefore warranted to investigate serological markers that could identify relevant subgroups within these autoimmune diseases to provide better treatment for patients. Throughout this thesis we focused on six different PTMs namely, nitration (Nt), citrullination (Cit), carbamylation (Ca), acetylation (Ac), malondialdehyde acetaldehyde adduct (MAA) and advanced glycation end-product (AGE) in the context of autoimmunity. In **Chapter 2**, we investigated anti-PTM antibodies targeting MAA- and AGE-modified proteins in a well-established RA cohort namely the Leiden Early Arthritic Cohort which contained information on ACPA as measured using the anti-CCP2 assay and anti-CarP. We observed that the presence of anti-MAA and anti-AGE antibodies identified a subgroup of RA patients that was seronegative for anti-CCP2, anti-CarP and Rheumatoid Factor (RF). Since HLA-DRB1*03 is associated with seronegative RA and anti-CarP antibodies in this disease subset, we sought to investigate the association of HLA-DRB1*03 with anti-MAA and anti-AGE in this study. We observed that anti-MAA was associated with HLA-DRB1*03 in the anti-CCP2 negative stratum, independent of anti-CarP. Anti-AGE followed the same trend but did not reach significance. We also observed that anti-MAA is associated with markers of inflammation in early arthritis in both RA and non-RA arthritis patients. Previous studies showed that anti-CarP was found to be associated with increased bone erosion in anti-CCP2 negative RA patients. We therefore investigated associations with radiographic joint damage in these patients. We observed

that in patients with ACPA-negative RA, anti-AGE is associated with radiological progression independent of anti-CarP, suggesting that this anti-PTM antibody could discriminate a different subgroup. Finally, correlation analyses were performed to investigate associations between anti-PTM antibody positivity and sustained drug free remission. We did not find any association of anti-MAA nor anti-AGE with sustained drug free remission, also not after stratifying for ACPA status. In conclusion, anti-MAA and anti-AGE antibodies are both prevalent in patients with RA, and other inflammatory rheumatic conditions, and although not specific for RA they each correlate with specific parameters. Anti-MAA associates with HLA-DRB1*03 in ACPA-negative (RA) patients independent of anti-CarP and associates with inflammation. Anti-AGE associates with HLA-DRB1*03 in patients with ACPA-negative RA and is associated with a worse radiological progression especially in patients with ACPA-negative and anti-CarP-negative RA. With this study, we have characterized a seropositive subgroup (anti-MAA and anti-AGE positive) within the heterogeneous group of patients with RA that have thus far been considered seronegative (ACPA-, anti-CarP and RF-negative).

Now that we have established that in RA, next to antibody responses against proteins modified by citrulline or homocitrulline by citrullination and carbamylation, respectively, also proteins modified by other PTMs could be targeted by autoantibodies, we further explored different autoimmune diseases. Autoimmune diseases such as SLE and AILD display a variety of autoantibodies targeting self-proteins. Therefore it is plausible that these patients harbor antibodies that bind specifically to PTM-modified proteins. In **Chapter 3** we investigated anti-PTM antibody reactivities against all six different PTMs (Nt, Cit, Ca, Ac, MAA and AGE), in SLE patients. SLE patients are known for their global loss of self-tolerance. It is therefore plausible anti-PTM antibodies will be present. Since the Leiden University Medical Centre is a tertiary referral center for SLE patients with neuropsychiatric manifestations (NPSLE), we had the unique opportunity to investigate this specific manifestation. Identifying patients with NPSLE in the complex disease course of SLE is challenging and it would be highly desirable to have serological biomarkers to support the diagnosis. In the whole SLE cohort we observed that anti-MAA, anti-AGE and anti-CarP levels and positivity were significantly increased compared to healthy controls. On top of that, levels of anti-MAA and anti-AGE negatively correlated with complement C3 and C4 levels and positively with erythrocyte sedimentation rate (ESR) pointing towards systemic inflammation. Interestingly, we observed that anti-MAA and anti-CarP were more common in patients with major NPSLE than in patients with other SLE organ manifestations. On top of that, anti-MAA and anti-AGE correlated with white matter volume (WMV) and total brain volume (TBV), and anti-CarP with white matter hyperintensity volume. Since WMV, TBV and white matter hyperintensity volume are objective markers for the involvement of the central nervous system in SLE, they provide objective evidence next to the multidisciplinary assessment to diagnose these patients.

All other NPSLE activity markers are more subjective, such as mood for example. Taken together, anti-PTM antibodies could aid in the diagnosis and/or subgrouping of (NP)SLE. Future studies should further establish the potential role of anti-PTM antibodies in (NP) SLE.

In **Chapter 4** we investigated anti-PTM responses against our set of six PTM-modified proteins in patients with AILD. AILD is a heterogenous group of both cholestatic and hepatocellular diseases, consisting of primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH) and overlap variants. PBC, PSC and AIH are often considered (auto)immune mediated diseases and AIH and PBC are characterized by the presence of autoantibodies and elevated total immunoglobulins. Additionally, autoantibodies are nowadays implemented in the diagnostic work-up but are not disease specific. We observed increased anti-PTM antibodies targeting Ca-, Ac-, MAA- and AGE-modified proteins in patients with AILD compared to healthy controls. Next to single positivity also positivity for more than one anti-PTM antibodies was analyzed. We observed that patients with AILD more frequently harbored at least one type of anti-PTM antibody compared to non-AILD and HCs. Since AILD consists of three subgroups, anti-PTM responses were assessed in these groups. Patients with AIH were significantly more often positive for anti-MAA, anti-AGE, anti-CarP and anti-Cit compared to patients with cholestatic liver disease (PBC and PSC). For patients with AIH, presence of anti-MAA and anti-AGE correlated positively with complete biochemical remission (CBR) at 3 months. In addition, anti-MAA, anti-AGE, and anti-CarP positively associated with achieving CBR at 12 months. Based on the discovery of multiple anti-PTM antibody positivity in patients with AIH, we attempted to discover the clinical relevance of harboring these multiple anti-PTM antibodies. After 3 months treatment, significantly more AIH patients with at least three anti-PTM antibodies had reached CBR. After 12 months of treatment, the difference was still significant. Overall, a trend toward significance for time to CBR (in years) was found in favor of multiple anti-PTM antibody positivity. In conclusion, anti-PTM antibodies are present in patients with AILD. Some patients are positive for multiple anti-PTM antibodies. Having three or more anti-PTM antibody responses is associated with a favorable response to treatment in AIH. A commentary was published based on **Chapter 4** (included in **Chapter 4**) questioning whether we were measuring anti-PTM antibodies or (also) measuring polyreactive IgG. In a reply on the commentary (included in **Chapter 4**) we clarified our methods explaining that in our experimental set-up we namely include mock-modified FCS to correct for reactivities towards FCS, reassuring that we are measuring IgG antibodies that specifically target PTM modified proteins.

Interestingly, many patients with autoimmune disease, including RA, SLE and AIH, benefit for B-cell targeted therapy suggesting a (pathogenic) role for B-cells and/or antibodies. However, what triggers these B-cells to become autoreactive is currently under debate.

In **Chapter 5** we shed light on PTM-modified proteins that could trigger the complement system which in turn may lead to clearance, chronic inflammation and/or autoimmunity.

In this context we investigated our set of six PTMs (Nt, Cit, Ca, Ac, MAA and AGE). Proteins (fetal calf serum) modified with these PTMs were previously used to establish IgG antibody reactivities in the RA, SLE and AILD cohorts in **Chapters 2, 3 and 4**. We have now combined the antibody profiles of RA, SLE and AILD against all six PTMs in **Chapter 5** in order to investigate combinations of anti-PTM antibodies occurring in autoimmunity. Interestingly, we observed that antibodies targeting Cit-, Ca-, Ac-, MAA- and AGE-modified proteins mostly co-occurred. To study how PTM-modified proteins could be involved in these chronic autoimmune diseases we set out to screen for plasma proteins that bind to PTM-modified proteins. Using mass spectrometry we could identify that from human serum it were mostly complement components that were binding specifically to PTM-modified proteins with the PTMs Ca, Ac, MAA and AGE compared to non-modified proteins. In order to verify direct binding and activation of complement to Ca-, Ac-, MAA- and AGE-modified proteins binding assays with purified complement components and complement activation assays were employed. We verified that complement indeed binds directly to the PTM-modified proteins, without the involvement of antibodies. Since the complement system entails three activation pathways (classical, lectin and alternative) we sought to allocate a complement pathway to each PTM-modified protein. We observed that Ca-, MAA- and AGE-modified proteins activate the classical pathway and verified that Ac-modified proteins activate the lectin pathway. The complement system is known for its clearance function but is also known to link innate and adaptive immunity through complement receptors. We therefore set out to investigate cells from whole blood and THP-1 macrophages as a model for more tissue resident cells. For this purpose we coupled PTM-modified proteins to beads using strep-biotin interaction to mimic long lived proteins that are frequently exposed to PTMs. Next, we incubated those beads with complement active serum to opsonize the beads with complement. We observed that leukocytes and THP-1 macrophages showed increased binding and uptake of complement opsonized PTM-modified protein coupled beads compared to non-opsonized PTM-modified protein coupled beads or non-modified protein coupled beads. At last, we had the opportunity to use data on single-nucleotide polymorphisms (SNPs) of complement genes in a set of RA patients in which we also measured anti-PTM reactivities. For this purpose we performed association studies between presence of anti-PTM antibodies and SNPs in complement genes known to be involved the activity of the complement system. 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 presence of anti-CarP. These data indeed suggest a link between complotype and development of anti-PTM antibody reactivity. With this study we emphasize that complement activation is triggered on several specific

PTMs, but clearly not all PTMs react similarly. In conclusion, PTM-modified proteins can bind and activate complement, which increases inflammation and phagocytosis, and may lead to development of anti-PTM antibodies.

As summarized and discussed in **Chapter 6**, the research described in this thesis shows that PTMs and anti-PTM antibodies associate with disease progression and treatment response. Additionally, detection of anti-PTM antibodies could be utilized to discriminate subgroups in several autoimmune diseases. Further studies should be employed to pinpoint potential diagnostic and prognostic value within each group. It has become apparent that some PTMs and anti-PTMs are involved in (chronic) inflammation, as we have observed associations between anti-PTMs and inflammatory markers throughout different cohorts. Whether these anti-PTMs are causing inflammation or are a result of (chronic) inflammation is to be explored in the future. We however established that complement proteins could directly bind to PTM-modified proteins to enhance clearance but that depending on the complotype this process could lead to (sustained) inflammation and autoantibody production. The research presented therefore urges further investigation of complement in the induction of anti-PTM antibodies *in vivo* by making use of complement deficient mice. Next to this fundamental research also studies into therapeutic opportunities are warranted. Strategies need to be developed to specifically deplete anti-PTM reactive B cells and also strategies to specifically inhibit complement activation driven by PTM. Our team has recently developed bispecific antibodies that with one antibody arm bind to PTM protein and with the other recruit an endogenous complement inhibitor to dampen complement activity on PTMs. This strategy would fit in the proposed interventions aiming for sustained immunological remission leading to sustained (drug-free) remission.

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