The value of rheumatoid arthritis autoantibodies in disease pathogenesis and treatment prognosis

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CHAPTER 8

Discussion

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting mainly small joints, associated with specific autoantibodies recognizing modified protein epitopes, termed anti-modified protein antibodies (AMPAs). Among these, anti-citrullinated protein antibodies (ACPA) targeting citrullinated epitopes are well-studied. Additionally, other AMPAs recognizing carbamylated, acetylated, and malondialdehyde-acetaldehyde modified proteins have emerged (1-3). Understanding AMPAs, particularly ACPA, has reshaped RA pathophysiology theories, linking them to genetic factors like HLA shared epitope (SE) alleles and environmental factors like smoking (4-6).

Although ACPA-positive RA has been associated with specific genetic and environmental factors, the exact mechanisms triggering autoantibody formation remain unclear. Research on other autoimmune diseases in diverse populations has uncovered unique interactions between genetic susceptibility and environmental triggers, exemplified by fogo selvagem in Amerindians(7), as described in **Chapter 2**. Investigating autoantibody responses across diverse populations and ethnic groups could unveil novel risk factors and disease mechanisms that play a role in the development of the autoantibody response and of the resulting autoimmune disease like RA.

To that end, **Chapter 2** explored the geoepidemiology of AMPAs (anti-CarP, anti-MAA, and anti-AcVim) in four ethnically diverse ACPA-positive RA populations from different continents: The Netherlands, First Nations (Canada), Japan, and South Africa. We observed evident differences in AMPA prevalence and levels across cohorts, in line with previous studies that reported prevalence of anti-CarP in non-Caucasian/non-Western populations (8, 9); no other data yet exists on population differences in anti-MAA or anti-AcVim. However, observed differences largely corresponded to variations in total IgG levels rather than the classic RA risk factors HLA SE, HLA DRB1*03, or smoking. In fact, we found no consistent associations between smoking, HLA SE alleles, and AMPA positivity across cohorts. Later studies also found no association of non-ACPA AMPAs with HLA SE and smoking in a clinically suspect arthralgia cohort (10). In another study, the association with HLA-SE and smoking with various AMPAs showed conflicting results, also when restricting the analysis only to anti-CCP2 positive RA like in our study: a positive association of smoking or HLA-SE was found with some anti-acetylated proteins and with some anti-carbamylated proteins but not with others (11). It is difficult at present to draw conclusions from this, and more research is needed to elucidate whether AMPAs associate with classic RA genetic and environmental risk factors, as is the case for ACPA.

Overall, despite the lack of association, our findings do tell us something interesting: while AMPAs are present in diverse RA populations across the world, population-specific risk factors do not seem to significantly shape their development. This implies that the development of a broad AMPA response, pathognomonic in seropositive RA, is the final common outcome of different pathways involved in disease. Work by our colleagues supports this theory in mice, where immunization with carbamylated proteins induces

development of antibodies recognizing both carbamylated and acetylated (self) proteins, and vice versa for immunization with acetylated proteins (12, 13). Anti-CarP and ACPA responses are also cross-reactive to a certain degree (13, 14), as are the B-cells producing them (15). In this way, different AMPA responses can originate from a common B-cell response that may lead to disease development, regardless of the original PTM or antigen this B-cell clone recognized, which may indeed vary greatly dependent on local exposures to environmental stimuli, microbiotic infections, or genetic susceptibility.

However, other factors must also play a role in shaping the AMPA response because cross-reactivity alone is not enough: 1) both AAPA and anti-CarP-responses are only partly cross-reactive with citrullinated antigens (3, 16) and 2) anti-MAA-antibodies display no cross-reactivity at all (17). There are multiple other processes that have been suggested to play a role in ACPA-producing B-cells surviving the various natural checkpoints that normally eliminate autoreactive clones. One promising mechanism is the selective introduction of V-domain N-glycosylation sites during T-cell aided somatic hypermutation (SHM) of the ACPA-reactive B-cell receptor that, contrary to SHM in other immune responses, apparently does not produce affinity maturation toward citrullinated antigens (18, 19). This may allow autoreactive ACPA B-cells, cross reactive to other PTMs, to escape negative selection by impairing the strength of their binding while still allowing the clones to remain constantly stimulated due to ever-present autoantigens, as demonstrated by presence and persistence of classically short-lived IgM ACPAs (20) and IgM AMPAs (**Chapter 4**).

Alternatively, or additionally, glycosylation of the B-cell receptor may lower the threshold for B-cell activation, preferentially allowing autoreactive clones to survive (21). These processes have so far exclusively been investigated in ACPA-specific B-cells; more research is needed to determine if the same mechanisms could be at play in B-cells reactive to the most recently discovered AMPA targets. Our results suggest that whatever the mechanisms to broad AMPA development, it follows a common path in patients with different ethnic and geographical backgrounds.

Chapters 3-5 extensively explored the AMPA response in RA and its implications for treatment outcomes. The autoantibody profile exhibits great diversity. AMPAs target variable numbers of distinct peptides with identical and divergent post-translational modifications and demonstrate significant heterogeneity in isotype usage (11, 22, 23). This breadth of the autoantibody profile likely arises from the breakdown of tolerance to multiple autoantigens and underscores the maturity of the humoral autoimmune response once RA manifests.

The diversity and breadth of the autoantibody profile raises the question whether this is associated with treatment outcomes. Early initiation of disease-modifying drugs (DMARDs) has improved clinical remission rates (24, 25), but not all patients

achieve sustained drug-free remission (DFR) – the closest approximation of disease cure available (26). Since autoantibody seropositivity is a poor prognostic factor for this treatment goal, we hypothesized that the breadth of the baseline autoantibody profile (**Chapter 3**), seroconversion to autoantibody-negativity (**chapter 4**), and changes in autoantibodies over time (**Chapter 5**) may set apart patients that do achieve early clinical remission or long-term sustained DFR. Additionally, we investigated, as previous publications have done, whether one specific AMPA, a combination of them, and/or a specific pattern, with its changes over time, have any prognostic value.

Chapter 3 demonstrated that a broader autoantibody profile at baseline was associated with a greater early response to treatment, parameterized by a greater decrease in DAS after 4 months of conventional DMARD therapy in the IMPROVED trial. Conversely, that same broad profile was linked to a smaller chance of achieving initial DFR during early drug tapering attempts. It did not impact chances of achieving long-term sustained DFR. In fact, baseline seropositivity for anti-CCP2 IgG was the only relevant factor associated with inability to achieve long-term sustained DFR.

Our results show that the breadth of the autoantibody response is mainly relevant for early treatment response. We also found a trend that the number of AMPAs present was inversely related to likelihood of achieving initial DFR. Analyses of the RETRO study by others support this trend: patients with a broad pattern of AMPA response relapse more often in the first year after DMARD tapering (27, 28). Reasons for the difference in effect size in our study lie in different definitions of the outcome and in our lack of a seronegative comparator group. Studies that dichotomize RA into seropositive and seronegative disease have not found an association with autoantibody status and early treatment response, again underlining the importance of specific characteristics of the autoantibody profile, over simple dichotomy (29). The association of seropositivity as a poor prognostic factor for long-term outcomes is well-established in literature, and our results align with that (26).

What our findings suggest is that the presence of multiple antibodies at baseline may be a marker of an active autoimmune response, characterized by reactivity against multiple PTMs, extensive isotype usage, and possibly ongoing activation (23). We propose that patients with this profile may be more susceptible to immunosuppression by methotrexate and prednisone, explaining their better early response. However, the importance of the baseline autoantibody profile diminishes over time, despite autoantibodies staying relatively stable over time (more on this in **Chapter 4**). At later time points closer to tapering, the breadth of the autoantibody profile did not provide additional insights into long-term outcomes.

Baseline anti-CCP2 IgG positivity was the only aspect of the profile to remain significant for long-term DFR. Multiple studies have demonstrated the enduring presence of ACPA

memory B cells in circulation, even in patients in clinical remission under conventional DMARDs (30-32). Additionally, persistent antibody positivity could also be due to bone marrow long-lived plasma cells. It is likely that the clinical anti-CCP-test is good indicator of long-lived autoimmunity and, compared to a broader autoantibody profile, therefor seropositivity for this indicates a subset of patients unable to successfully drug-tapering in the long run.

Chapter 4 builds upon previous findings to explore whether autoantibody levels exhibit changes over time and whether this change confers any prognostic advantage. It examines the concept of 'immunological remission', wherein conversion from seropositive to seronegative status for RA-associated autoantibodies could potentially signify a state of more profound disease remission than the absence of symptoms or synovitis (33). The study determined the frequency and significance of seroconversion for various RA-associated autoantibodies in a cohort aiming for drug-free remission, and the impact of seroconversion on successful drug discontinuation.

In the IMPROVED study, longitudinal measurement of fourteen autoantibodies revealed modest rates of seroconversion to negative, yet this did not correlate with favorable outcomes for sustained drug-free remission (SDFR). Despite efforts to establish stricter criteria for seroconversion, such as complete or multiple autoantibody seroconversion, no association with SDFR was found (supplementary material). We conclude that autoantibody seroconversion does not effectively identify patients in a form of immunological remission wherein underlying autoimmune processes have been quenched. Investigations by colleagues in the Leiden Early Arthritis Clinic cohort found similar results for anti-CCP2 IgG/IgM and RF IgM seroconversion (34). Other studies support this notion on a cellular level: patients in clinical remission, similarly defined as in our study, retain an activated phenotype of ACPA-specific B-cells, suggesting that clinical remission is simply a state of suppressed inflammation in which immunological processes persist (30, 35). Currently, signals of low inflammatory load, such as DAS or ultrasound findings, appear to be better predictors of successful drug tapering than markers of humoral autoimmunity loss.

This study pioneered the attempt to operationalize immunological remission as a measurable entity and assess its relevance to drug tapering success, concluding that seroconversion to negative is not associated with the success of drug discontinuation and is not be a meaningful biomarker in tapering decision-making. However, it does not refute the possibility of the existence of immunological remission in RA. Kristyanto et al. also investigated ACPA-specific B-cells in the pre-disease phase in 'at risk' arthralgia patients, and found that ACPA-expressing memory B-cells are already present and produce phenotypically identical ACPAs as in established RA patients, although the B cells do not yet show an activated phenotype (35). This subset proliferates and activates as patients progress to arthritis, and, as of yet, have not been found to revert to their quiescent state,

even in states of clinical remission. This quiescent state, or other cellular phenotypical states of ACPA B-cell deactivation, may be the true immunological remission that indicates which patients may safely taper DMARDs. The first results of the long-awaited ASCARA trial suggest that indeed, ACPA-expressing B-cells can be modulated by a CTLA-4 agonist (abatacept) towards a deactivated phenotype compatible with immunological remission (36). Whether this form of immunological remission leads to improved clinical outcomes or ability to taper DMARDs remains to be seen.

Chapter 5 further explores the potential of autoantibodies as biomarkers for disease severity and treatment outcomes in RA, focusing this time on changes in levels within seropositive subsets. We had hoped that serological changes in RA might prove to be an easily accessible biomarker for the future disease course, as is the case in other autoimmune diseases. A substantial drop in autoantibody levels could for example be a clinically tangible prognostic tool if it precedes successful drug-free remission. Additionally, such an association and its relationship with immunosuppression and disease activity could shed light on autoimmune B cell response mechanisms in RA. Studies documenting the relationship between fluctuations in autoantibodies and disease activity have been conflicting (37-39) possibly because they did not consider the influence of immunosuppressive treatment on level changes and disease activity. Additionally, **Chapter 4** has shown that seroconversion to negative, which of course requires a change in levels, had little prognostic value. However, seroconversion happened mostly in patients whose antibody levels were already close to the cut-off for positivity. It is possible that robust changes in levels hold more prognostic information that merely fluctuations around a cut-off.

Therefore, we conducted a longitudinal characterization of changes in RA-associated autoantibody levels over time. Our results revealed no significant association between autoantibody changes and disease activity, functional status, treatment response, or long-term outcomes like DFR and radiographic progression. Instead, the observed changes in autoantibody levels appear to primarily reflect the effects of immunosuppressive therapy, rather than indicating disease-specific clinically relevant processes. Consequently, monitoring autoantibody levels over time appears to offer limited value in clinical settings.

However, it does teach us something about RA pathophysiology. Despite both autoantibody levels and disease activity decreasing significantly under anti-inflammatory therapy, RA persists in the long term. We hypothesize that this may reflect differences in longevity and place of residence of the autoantibody-producing cells. It seems plausible that synovial inflammation provides a niche that promotes the survival of ACPA-producing plasma cells (40), which falls away or is eliminated when treatment is initiated or intensified, thereby decreasing autoantibodies. However, the autoantibody-producing plasma cells may never be fully eradicated from their bone marrow niches, even in the absence of clinical symptoms, as previously discussed.

Following in the footsteps of Chapter 5 and of other groups evaluating multi-biomarker disease activity scores and imaging markers, Chapter 6 explored a marker of possible residual subclinical inflammation: circulating calprotectin. Calprotectin, also known as S100A8/A9 or myeloid-related protein (MRP) 8/14, is a heterodimeric soluble protein complex released by leucocytes during infectious and inflammatory conditions. Traditionally used in inflammatory bowel disease diagnostics (as fecal calprotectin measurements), calprotectin differs from conventional acute-phase reactants like C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) as it is locally released at sites of inflammation (41). In RA, calprotectin correlates with disease activity markers and synovial inflammation, is associated with erosive progression, and may predict response to (especially biological) DMARDs (42, 43). Calprotectin levels show special promise in juvenile idiopathic arthritis, where they correlate with disease activity and relapse risk after DMARD discontinuation (44, 45). If such an association exists in RA, it could serve as a practical biomarker for predicting disease flare after tapering, aiding risk stratification and clinical decision-making.

Chapter 6 evaluated circulating calprotectin's role in predicting flare upon DMARD tapering in two independent cohorts (IMPROVED and RETRO study) and found that higher calprotectin levels were associated with increased flare risk within 12 months post tapering in the RETRO study, and that this improved upon conventional flare prediction using clinical parameters. There was a smaller, non-significant effect in the IMPROVED study, possibly because of differences in disease duration, outcome measures (DAS44 vs DAS28-ESR), definitions of remission stability, and treatment use (prednisone in IMPROVED, biologicals in RETRO). Potentially, calprotectin might not be able to discriminate residual disease activity when the definition of remission is very strict, as was the case in the IMPROVED study (DAS44<1.6, compared to DAS28-ESR<2.6 in the RETRO study); however, sensitivity analyses in the IMPROVED study using RETRO's remission definition did not change the results. Even more puzzling is the fact that a group in Nijmegen has conducted a very similar investigation of calprotectin in DFR in the DRESS study, as has another group in France, in the STRASS study, and found no value of calprotectin for predicting successful anti-TNF tapering (46, 47). The cohorts in these studies are similar to especially the RETRO study regarding patient characteristics and design, including: long-standing disease, concomitant conventional synthetic DMARD use (particularly prednisone), minimal 6 months remission before tapering, previous DMARD use, and current use of TNFα inhibitors. Main differences are in the definition of remission (RETRO: DAS-28-ESR<2.6; DRESS: DAS28-CRP<3.2; STRASS: DAS28<2.6), and in the definition of RA (respectively: EULAR 2010; ACR 1987/ EULAR 2010/clinical diagnosis; ACR 1987). However, all of these disease activity indices have been shown to correlate well(48), and given the disease durations in these cohorts (all long-standing disease), differences in selection criteria are unlikely to explain the disparity in calprotectin results. These inconsistent findings across all cohorts challenge calprotectin's application; it is unclear whether the results in the RETRO study may be a

spurious finding. Further research is needed to examine its utility in clinical practice, for doing so could improve RA management, offering personalized strategies for achieving drug-free remission while minimizing disease flare risks.

This thesis also delved into the pathophysiology of a different type of autoimmunity; namely, iatrogenic autoimmune reactions induced by immune checkpoint inhibitors (ICIs). Ipilimumab, a CTLA-4 inhibitor and the first ICI, caused a paradigm shift in cancer immunotherapy when first introduced in 2011 in the field of metastatic melanoma, and since then, multiple ICIs have improved the previously dismal prognosis of patients with various types of cancer. However, this came at the cost of serious immune-related adverse events (irAEs), described to date in virtually every organ system, including the joints (49). The mechanism of these reactions lie in ICIs inhibition of negative costimulatory signals to T cells which enhance antitumor T-cell responses (50). Because this mode of action is not antigen-specific, ICIs may also (re)activate otherwise dormant autoreactive T cells. We hypothesized that this expansion would be accompanied by T cell–dependent activation of autoreactive B-cells and autoantibody production, which in turn may be associated with more frequent irAEs and with better antitumor responses, as has been reported for changes in the T-cell repertoire (51-53).

In Chapter 7, we observed that ipilimumab treatment induced autoantibody development in a significant portion of melanoma patients. There was a trend suggesting an association between autoantibodies and irAEs under ipilimumab treatment, and a subanalysis in only irAEs related to the development of any of the antibodies measured was significant for this association. Our study was unfortunately underpowered to directly investigate the association between antibody development and the irAE in the organ system for which that antibody has conventional diagnostic use. However, patients who developed autoantibodies, particularly thyroid autoantibodies, showed minor survival and response benefits.

We gave special attention to thyroid autoimmunity in our study. As we found, previous research shows that ipilimumab treatment can induce thyroid autoantibodies, even without overt thyroid dysfunction (54-57). Thyroid autoantibodies are a special case in autoimmunity because they occur commonly in populations without overt thyroid disease or non-thyroid concomitant autoimmune disease (58-64). Additionally, mutations in CTLA-4, a key regulator in autoimmunity (65) and the target of ipilumumab, contributes to the genetic susceptibility to thyroid autoantibodies development, possibly independently of manifest thyroid autoimmunity (66, 67). Lastly, thyrocytes, which are not classically antigen presenting cells, are able to present MHC class II molecules and thyroid antigens in autoimmune thyroiditis in vivo (68), which are recognized by T-cells which in turn activate B-cells to produce autoantibodies.

Interestingly, in our study, the development of thyroid autoantibodies predisposed euthyroid ipilimumab-treated patients to subsequent thyroid dysfunction under anti-PD-1 therapy. Induction of thyroid autoimmunity by anti-PD-1 therapy in patients with pre-existing thyroid autoantibodies has been described previously (69-71), and confirmed recently (72, 73). This probably rests in the vital role of PD-1/PD-L1 axis in maintaining thyroid tolerance (74): in autoimmune thyroiditis, circulating levels of PD-1 positive T-cells are enriched. In response, thyrocytes have increased levels of PD-L1 expression, intent on keeping budding autoimmunity in check, and possibly explaining why autoimmune thyroiditis is generally a slowly progressive illness. In PD-1 inhibition with ICIs, however, this attenuation falls away, and could explain why thyroid antibodies and overt thyroid autoimmunity develops. We suggest that in patients who have been treated with previous CLTA-4 inhibition, this process may be primed and occur more frequently, though future studies are needed to elucidate the mechanism.

In our study, autoantibody development – thyroid autoimmunity in particular – was also associated with (minor) survival and response benefits. These results are in line with another longitudinal study in nivolumab (PD-1 inhibitor) treated lung cancer patients, wherein development of auto-antibodies (anti-nuclear antibodies, anti–extractable nuclear antigen, and/or anti–smooth muscle antibodies) was predictive of irAEs development and survival (75). It is likely that this autoimmunity is a marker for effective ICI-induced immunogenicity, reflecting enhanced immunity that targets both tumors and self-tissues.

Thyroid autoantibody production in particular was most strongly associated with irAEs, survival, and therapy response. One other study noted that thyroid-autoantibodies are associated with overt thyroid dysfunction, and that thyroid irAEs are associated with better survival, but a direct association between autoantibodies and survival is unfortunately not reported(70). Whether there is a specific mechanistic link between anti-thyroid immunity, in the form of autoantibodies or irAEs, and anti-tumor immunity is unknown, and more studies are needed to validate this association.

It is worth noting in the larger context of this thesis that we found that classical rheumatoid arthritis autoantibodies (anti-CCP2 and RF) only developed in 3 out of 133 patients who were seronegative at baseline, and that in these patients, no one developed a rheumatic irAE. Conversely, in patients who did develop a rheumatic irAE, no one developed autoantibodies. A recent meta-analysis of 372 anti-PD1 or anti-CTLA-4 treated patients with various forms of cancer confirms that seropositivity in “rheumatoid arthritis–like” irAEs is rare: 9%, compared to the 70% in classic RA (76). This begs the question: how similar are the mechanisms that lead to induction of ICI rheumatic illness compared to those that lead to RA? Comparisons between RA populations in **Chapter 2** showed striking similarity in the prevalence of AMPAs, despite vastly different genetic and environmental backgrounds, pointing to a common final

pathway. Studies that attempt to discern the immunogenetics of patients that develop rheumatic irAEs or autoantibodies have shown conflicting results. One study showed that HLA SE was enriched in patients with ICI-induced rheumatic irAEs compared with ethnically matched healthy controls, with similar rates of HLA-SE heterozygosity as matched RA controls (77). This is a surprising finding as, in line with our study, very few patients with rheumatic irAEs were seropositive for anti-CCP2, whilst considering the strong association of HLA SE and ACPA in RA, one would perhaps also have expected HLA SE-positive oncology patients with rheumatic irAEs to develop ACPA. A later study was unable to replicate the association of rheumatic irAEs with SE, but was able to show that ACPA in patients with rheumatic irAEs was different from established RA patients, with lower ACPA levels and less epitope usage (78). Currently, there is too little data available to draw conclusions about the interplay of autoantibodies, genetic risk, and immune dysregulation in rheumatic irAEs. However, the importance of this cannot be understated. At its basis, irAEs in ICI treatment can serve as a human model for classic autoimmune disease. Identifying the inciting events and risk factors that lead to autoimmunity could shed light on early stages of pathogenesis of diseases like RA.

CLOSING REMARKS

This chapter provides a summary and discussion of the findings of the studies described in this thesis. First, to better understand the pathogenesis of RA, we investigated the relationship of genetic and environmental risk factors with the AMPA profile in different populations of RA, and found epidemiological evidence for a final common pathway in AMPA formation. We went on to further characterize this AMPA profile over time, and showed that baseline composition, but not changes over time, were relevant for RA prognosis. The outcome of drug-free remission in particular was highlighted as a potential proxy for RA cure, but our results suggest that the holy grail of immunological remission lies beyond changes in serological measurements of AMPAs. Calprotectin's use as clinical decision-making tool in attempting drug-free remission requires further investigation. Lastly, we showed that autoantibodies are formed during treatment with ICIs, and that a subset of these are possibly relevant for treatment outcomes.

Throughout this chapter, I placed results in context of what is known, attempted to address some of the major challenges of research in autoantibodies, and proposed future avenues of research to build upon what we discovered. Although the studies in this thesis provide us with new insights in different aspects of autoantibodies and rheumatoid arthritis, more questions were raised than were answered, as is inherent to research. I hope that this thesis contributes to new understandings of disease mechanisms in RA and autoimmunity, and that these findings may, following expansion by other clinical and basic science studies, translate into new approaches for prognostic evaluation and more targeted treatment of rheumatoid arthritis.

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