How is autoimmunity against citrullinated proteins regulated?
Cantaert, T.

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CHAPTER 10

General discussion
In this thesis, we investigated which mechanisms could contribute to the regulation of autoimmunity against citrullinated proteins in RA. The data presented here, together with other studies, indicate that a combination of factors is involved in the break of tolerance towards citrullinated proteins. In this general discussion, we highlight the major findings of the thesis and summarize the current viewpoints on the development of the ACPA response. We put special focus on intrinsic defects in B cell tolerance mechanisms (Chapter 1 and 2), citrullinated antigens (Chapter 3-6), and immune interactions implicated in the development of the autoimmune response (Chapter 7-8).

B cell tolerance

Autoantibody production is the serological hallmark of the end stage of complex autoimmune processes, often initiated by intrinsic defects in B cell tolerance mechanisms. The impact of defective B cell tolerance has primarily been studied in experimental SLE models. Abnormal expression or function of key signaling molecules and B cell growth factors, dysregulation of cytokine homeostasis with key B cell effects, and perturbations in B cell developmental subsets not only lead to autoantibody production, but also to disease in many mice models (1-7). The relevance of these findings for human autoimmune diseases has recently been demonstrated by Nussenzweig and Wardemann, who demonstrated elevated percentages of self-reactive and poly-reactive B cells in different stages of B cell development in SLE patients compared to healthy controls (8-11). This clearly indicates that human SLE is characterized by intrinsic defects in B cell tolerance mechanisms. To what extent these defects are either necessary or sufficient for the initiation of SLE is not yet clear. Part of the answer may be provided by our studies on ANA induction during TNF blockade in spondyloarthritis (SpA) patients (chapter 1). Although poly- and self-reactivity in different B cell compartments has not yet been analysed in this disease, the absence of autoantibodies strongly suggests intact B cell tolerance checkpoints in SpA patients. Our studies reveal 3 interesting features of B cell autoimmunity in these patients. Firstly, a large majority of the SpA patients treated with infliximab develop ANA, indicating that they have indeed autoreactive cells in their B cell pool. Secondly, the induction of these autoantibodies is related to excessive exposure to nuclear antigens but not to factors such as type I IFN, BAFF, and APRIL (chapter 1 and unpublished observations) indicating that quantitative (and possibly also qualitative) alterations of the antigens can trigger humoral autoimmunity. Finally, this autoimmune response was restricted to IgM and disappeared upon interruption of TNF blockade (12). This indicates that either the presence of intrinsic B cell tolerance
checkpoints in SpA, or the absence of downstream immunological mechanisms involved in the maturation of the B cell response, prevents the development of a full-blown, pathogenic humoral autoimmune response. We are currently investigating the latter hypothesis by evaluating the effects of TNF blockade on alloantibodies in an experimental model as well as in humans. Also in human RA, it has been shown that both central and peripheral checkpoints are defective (13). Although it is likely that this may favor the development of humoral autoimmunity, it remains unclear to which extent this determines the ACPA response. The fact that patients with SLE and Sjögren’s syndrome rarely develop ACPA indicates that intrinsic defects in B cell tolerance are not sufficient to initiate this autoimmune response. On the other hand, ACPA are found in only 50-70% of the RA patients and it would thus be of major interest to know if there are differences between intrinsic B cell tolerance mechanisms in the seropositive and seronegative subset of RA. One crucial question to be answered here is at what stage B cell immunity against citrullinated proteins is broken. Is it in the bone marrow, due to defects in central tolerance? Or is it in the peripheral compartment, due to either defective negative or abnormal positive selection processes (14-16)? Or is the autoimmunity acquired at a later stage during somatic hypermutation in the germinal center (17)? The direct identification of the ACPA+ pro/pre/emigrant/naïve or memory B cell remains crucial to elucidate these questions. We are currently developing new molecular tools to assess this issue.

Both intrinsic and extrinsic factors can influence B cell tolerance. For example, the strength of B cell receptor signaling is crucial in regulating thresholds for human B cell tolerance (18). It has been shown that a missense polymorphism in the protein tyrosine phosphatase PTPN22 gene is associated with ACPA+ RA (19). This protein is an important regulator of B and T cell receptor signaling. On the other hand, BAFF is one of the extrinsic factors regulating B cell autoreactivity by rescuing self-reactive B cells from cell death (6;20). BAFF serum levels are elevated in RA patients compared to healthy controls (21) and in synovial tissue of RA patients compared to SpA patients (chapter 7). Similarly, type I IFN levels are elevated in SLE and are associated with serum levels of some autoantibodies (22). An abnormal type I IFN signature has also been reported in a subset of the RA patients, but we demonstrated that this factor does not determine the ACPA response (chapter 2).

Taken together, these data confirm the concept that B cell autoimmunity is a ubiquitous phenomenon in humans (10) and suggest that defective B cells tolerance is necessary but not sufficient to initiate a full blown ACPA autoimmune response. We have now initiated detailed studies of intrinsic B cell tolerance to compare RA with SpA and healthy individuals.
Citrullinated antigens

Our study on the ANA response during TNF blockade in SpA emphasizes the importance of the antigen exposure in the break of immune tolerance. An additional aspect, which is crucial in ANA as well as in ACPA responses, is the qualitative change in the antigen by posttranslational modification. Citrulline is a non-standard amino acid which by itself is not sufficient to constitute an immunologic epitope. In addition, citrullinated proteins are not specific for RA, but are equally present in other inflammatory conditions and in healthy individuals. In contrast to the findings in our SpA model for anti-nuclear autoimmunity (chapter 1), it is thus not merely the availability or amount of citrullinated protein that determines the ACPA response. A crucial question here remains if well-defined citrullinated sequences are only present in RA and are able to initiate the ACPA response. In chapter 3, we confirm the presence of RA-specific citrullinated epitopes in the synovial tissue and indicate that they are probably generated by the enzyme peptidyl arginine deiminase type 2 (PAD2). On the contrary, we could not find evidence confirming the proposed hypothesis that polymorphisms in PAD4 would lead to the induction of ACPA through increased or altered activity of the PAD4 enzyme, which would generate more citrullinated proteins ((23) and chapter 4). One challenge for future research remains the biochemical identification of specific citrullinated epitopes which are specific for RA and which are not merely a target for ACPA, but are also able to induce the ACPA response.

In this thesis, we specifically investigated citrullinated proteins in the synovial tissue because this is the major target organ of the disease, and because ACPA are produced locally in the inflamed RA synovium. However, it remains to be proven that the immune response against citrullinated proteins is initiated here. In chapter 5 we demonstrated the presence of citrullinated proteins in the lung tissue of RA patients. As a gene-environment interaction has been observed between smoking and ACPA+ RA (24), one possibility is that tolerance against citrullinated epitopes is abrogated in the lung. Alternatively, smoking has a strong adjuvant effect as suggested by the association with other, non-citrulinated autoantibody responses as well (25;26). As described in detail in chapter 6, immunoreactive proteins are not per definition autoantigens. Accordingly, not all citrullinated proteins or citrullinated epitopes are autoantigens, as the recognition by ACPA is not only determined by the citrulline residue but also by the surrounding amino acids. This has elegantly been demonstrated by the identification of the epitopes targeted by ACPA within fibrinogen (27). Moreover, the fact that a citrullinated epitope is recognized by ACPA does not allow the discrimination between an antigen that is able to induce the humoral
autoimmune response and an antigen that is merely a secondary bystander target of the ACPA response. One could expect from other autoimmune systems that the primary citrullinated epitope which is responsible for the specific induction of ACPA in RA should have specific characteristics: intracellular localization (28), the ability to promote self-perpetuating interactions between B and T cells and reciprocal T cell-B cell diversification (29;30) and the ability to lead thereby to epitope spreading to other citrullinated or non citrullinated proteins (such as rheumatoid factor). This implies that, beside the characteristics of the antigen as such, the immunological interactions between autoreactive lymphocytes may be crucial for the development of a full-blown ACPA response.

**Immunological interactions between autoreactive lymphocytes**

As indicated, interactions between autoreactive B and T cells and reciprocal T cell-B cell diversification plays an important role in pathogenic autoimmune responses. Studies in a variety of chronic inflammatory diseases, including RA, suggest that such T-B cell interactions not only occur in lymphoid organs but also in inflamed tissues characterized by ectopic lymphoid neogenesis. This leads to the hypothesis that synovial lymphoid neogenesis in the inflamed RA synovium may promote or even initiate humoral autoimmune responses such as RF and ACPA. Interestingly, in chapter 7 we show that the presence of synovial lymphoid neogenesis is not restricted to ACPA+ RA or RF+ RA and not even to RA as such as we observe similar percentages of samples with synovial lymphoid neogenesis in inflammatory rheumatic disease controls such as SpA. Moreover, synovial lymphoid neogenesis tended to associate with lower synovial fluid ACPA levels. Taken together with our study showing lower serum ACPA levels in patients with lymphoid neogenesis (31), this could indicate that the installment of germinal center checkpoints in the inflamed tissue could help to restrict rather than promote the induction of humoral immune responses to multiple autoantigens that are formed/released during chronic inflammation (7;17;32-34).

T cells may also be involved in the break of immune tolerance towards citrullinated proteins as ACPA are class-switched antibodies (mainly IgG1 and IgG4) directed against a peptide epitope. In addition, the genetic association of ACPA+ RA but not ACPA- RA with specific HLA-DRB1 alleles (the shared epitope) (chapter 4 and (35)) supports the concept that T cells are involved in this disease subset and, eventually, in the ACPA response. In chapter 8, we provided molecular evidence for the role of synovial T cells in ACPA+ RA. We observed strong alterations of the complementarity
determining region-3 length distribution (CDR3-LD) in synovial T cells of ACPA+ RA, but not ACPA- RA or SpA. These CDR3-LD alterations were not associated with marked quantitative expansions of the clonal T cell size nor paralleled by B cell clonal expansions. Interestingly, the T cell clonal alterations were inversely associated with synovial lymphoid neogenesis, which is more frequently observed in ACPA- than ACPA+ disease (chapter 7 and (31)). These observations could indicate that synovial lymphoid neogenesis is not contributing to the CDR3-LD alterations by facilitating T-B cell interactions.

Two important questions still remain to be elucidated: Which subset of T cells are clonally restricted and is this (auto)antigen dependent? If the T cell response is antigen dependent, this could either be a citrullinated antigen or a non-citrullinated epitope of a larger protein complex of which a distinct citrullinated part is recognized by the B cell receptor, thereby allowing adequate antigen-specific T-B cell collaboration. A citrullinated peptide of vimentin, but not peptides derived from fibrinogen, showed higher binding affinity to the shared epitope HLA-DRB1 (36;37) compared with its native counterpart. In addition, T cell reactivity towards citrullinated fibrinogen has recently been shown in HLA-DR4 transgenic mice, but has never been proven in humans (38). An alternative hypothesis is that the TCR repertoire is shaped by the HLA-DRB1 molecule itself (39) or by homeostatic proliferation which can also lead to marked contractions of both the naïve and memory T cell repertoire in RA (40;41). Indeed, RA T cells display features reminiscent of accelerated senecce both in the naïve and memory pool indicating altered T cell homeostasis in RA (42). In this respect, the involvement of T cells in ACPA+ RA could be related to specific T-B cell interactions as well as to other mechanisms. As the expanded clonotypes are not detected in peripheral blood, we and others are working on more reliable synovial tissue cell extraction methods and further optimalisation of molecular techniques to provide insights in these questions in the near future.

Future directions

Generation of B cells with a high affinity B cell receptor does normally not occur in peripheral tissues such as the synovial membrane or the lung but in the secondary lymphoid organs. Our studies suggest that this holds true even in the presence of ectopic synovial lymphoid neogenesis. Accordingly, lymph nodes changes precede the development of arthritis in animal models of arthritis (43;44). A related issue is that plasma cells, the cellular source of the secreted ACPA, normally home to bone marrow rather than to peripheral tissues. It is also not yet known which type of plasma cells is responsible for ACPA production. Do they originate from follicular
B-2 B cells as suggested by the high-affinity interaction with the antigen? Or are they produced by marginal zone B-2 B cells as has been observed for RF producing plasma cells in mouse models (17)? Moreover, it is not known if the ACPA are produced by long-lived plasma cells or rather by short-lived plasmablasts (45). After B cell depletion by anti-CD20 monoclonal therapy ACPA IgG levels decreased more than total IgG levels but did not disappear from circulation (46;47), indicating that they are at least partially produced by long-lived plasma cells which could reside in the bone marrow or the inflamed joint (48). On the other hand, this also shows that their production may be dependent on the constant generation of short lived plasma cells from CD20+ B cells. In that respect, a major research focus for better understanding of the ACPA response is to combine immunological data from the inflamed joint with analysis of lymphoid tissue such as lymph nodes and bone marrow.

A second crucial issue for better understanding of the initiation and regulation of the ACPA response and the role of ACPA in RA, is the development of appropriate animal models. Classical arthritis models do not display an ACPA response even though citrullinated proteins are present in the inflamed joints (49). Detection of specific ACPA reactivity in mice sera is complicated by the observation that the commercially available ELISA using cyclic citrullinated peptide does not show the same specificity as it does for human samples. Therefore, a negative control using the non-citrullinated peptide should be included (50). One other major caveat is the use of in vitro citrullinated proteins both for preparation of the immunogen as well as for the detection of the antibodies, as in both preparations the rabbit PAD2 enzyme is present. Immunization generates mouse anti-PAD2 antibodies which can be detected by the ELISA coated with PAD2 only and which give false positive anti-citrulline results on ELISAs coated with the in vitro citrullinated antigen still containing the PAD enzyme (Cantaert T, unpublished observations and (51)). In many studies, this important bias has not been taken into account (38;52). One further requirement for a solid and reliable animal model to study B cell tolerance towards citrullinated epitopes is the use of self proteins rather than alloantigens for immunization. Almost all published studies used citrullinated collagen or fibrinogen from a different species to study the ACPA response, but this response is then alloimmune and not autoimmune. Only one study addressed this issue and indicate that immunization of rats with citrullinated but not native rat fibrinogen induced a genuine autoimmune response, even though a secondary immune response could not be elicited (51). Taken together, these mouse and rat data lead to the intriguing hypothesis that rodents may be less susceptible than humans to autoimmunity against citrullinated proteins. The use of human transgenic animals, such as the HLA-DR4 transgenic mice, may partially circumvent this issue. We are currently investigating the use of even more ‘humanized’ models such as collagen induced arthritis in rhesus monkeys and Rag2+/−γc−/− mice engrafted with human
cord blood hematolymphoid cells (53;54). The development and validation of such a model may not only allow to address in more detail the question of present thesis, what regulates the autoimmune response against citrullinated proteins, but may also allow to address the other major issue: are ACPA pathogenic or not?

In conclusion, we do not yet fully understand what regulates the autoimmune response against citrullinated proteins. However, we and others showed that, in parallel with other autoantibody systems, multiple factors are involved in the development of the ACPA response, including intrinsic defects in B cell tolerance, specific features of the citrullinated antigens, and immunological factors such as the shared epitope and T cell involvement. On the other hand, type I IFN, PAD polymorphisms, or ectopic lymphoid neogenesis do not appear to play a role in the ACPA response. Novel molecular tools to analyze B cell tolerance in RA, study of lymphoid compartments such as the lymph nodes and the bone marrow, and the development of relevant and reliable experimental models are important directions for future research.


12. De Rycke L, Baeten D, Kruithof E, Van den Bosch F, Veys EM, De Keyser F. Infliximab, but not etanercept, induces IgM anti-double-stranded DNA autoantibodies as main antinuclear reactivity: biologic and clinical


37. Hill JA, Southwood S, Sette A, Jevnikar AM, Bell DA, Cairns E. Cutting edge:
the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. J Immunol 2003; 171(2):538-541.


