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**Title:** Identification and characterization of anti-citrullinated protein antibody (acpa)-producing b cells in patients with rheumatoid arthritis (ra)
**Issue Date:** 2017-04-18
Chapter 7

Summary and discussion
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This thesis describes the characterisation of citrullinated antigen-specific B cells in patients with rheumatoid arthritis (RA). These cells express and secrete anti-citrullinated protein antibodies (ACPA), which represent the most specific autoantibody system in RA. We started out by identifying ACPA-producing B cells in peripheral blood. FACS sort followed by culture showed that these cells are not confined to the memory B cell subpopulation, as circulating plasmablasts/cells were found that spontaneously produced ACPA in culture (chapter 2). Further characterisation showed an increased frequency of ACPA-secreting plasma cells in synovial fluid (SF), i.e. at the site of inflammation, which harbours the capacity to form a survival niche in culture that promotes sustained (auto-)antibody production (chapter 3). Next, ACPA-expressing B cells were identified directly after isolation using a streptavidin tetramer-based flow cytometry staining technique developed in chapter 4. Furthermore, we provided evidence for altered development of ACPA-producing B cells, as these cells were found to express lambda light chains (LC) more frequently than non-autoreactive B cells (chapter 5). Finally, we investigated CD28 expression on ACPA-expressing B cells and total B cells in RA patients as relevant molecule for an enhanced live span of long-lived plasma cells (chapter 6).

The presence of ACPA in patients with RA is associated with a severe disease phenotype[1]. To better understand the underlying immune response against citrullinated antigens, characterisation of ACPA-expressing B cells is required. So far, studies showed that ACPA are different compared to antibodies against recall antigens since ACPA, for instance, have low avidity and show only limited avidity maturation[2, 3]. Furthermore, several studies show that ACPA can promote inflammatory responses[4-10]. In addition, it was found that ACPA-IgG are more extensively glycosylated in their Fab region compared to other IgG[11]. Together, these data suggest that ACPA-producing B cells could be different in molecular properties compared to other B cells. Therefore, this thesis focuses on the characterisation of ACPA-expressing B cells in patients with RA. We hypothesise that a better understanding of the phenotype and functional characteristics of these cells can lead to the development/identification of new therapeutic targets to prevent development or to eliminate these cells, which could lead to a less severe disease.

A major challenge hampering studies on ACPA-expressing B cells was, prior to the start of this thesis, the absence of a method to directly identify and visualize these cells on a single cell level. Therefore, we set out to develop an appropriate method to achieve this aim. Previous data from another research group had shown that ACPA production was detectable upon culture of mononuclear cells from SF but not from peripheral blood[12]. Despite this observation, we
first focused our research on peripheral blood, as peripheral blood is easier to obtain than SF, and as the detectability of ACPA depends on the assay used. As described in chapter 2, we isolated the total CD19-expressing B cell population from peripheral blood mononuclear cells (PBMC) and cultured it under broad stimulating conditions. We could indeed measure ACPA production by B cells isolated from peripheral blood of RA patients but were unable to detect this production if B cells were isolated from ACPA-negative RA patients or healthy donors. In parallel, another group[13] also provided data indicating the presence of ACPA producing B cells in peripheral blood. We extended our research to discriminate between subpopulations of B cells based on the surface expression of CD20 and CD27 to define naïve B cells, memory B cells or plasmablast-/cells (PB/PC). Upon stimulation, we could detect ACPA-production by memory B cells and, in addition, could detect spontaneous production of ACPA by circulating PB/PC. These data supported the hypothesis that the citrullinated antigen-specific immune response is an ongoing, continuously active immune response, which would be different from, for example, a resting memory B cell response against recall antigens.

Since ACPA titres are increased at the site of inflammation compared to peripheral blood[14], one could hypothesise that the frequency of autoreactive B cells is also higher. As we were successful in detecting ACPA in culture indicative of the presence of ACPA-secreting B cells in peripheral blood, we therefore investigated the presence of these B cells in SF. B cells had previously been isolated from synovial tissue (ST) followed by cloning of the BCR. This approach had revealed a substantial frequency of citrullinated antigen-specific B cells in this compartment[15, 16]. Applying our culture technique developed in chapter 2, we indeed identified an increased frequency of spontaneously ACPA-secreting cells in the synovial fluid (chapter 3). It remained unclear, however, whether this high frequency is due to migration, retention, local generation or prolonged survival of ACPA-secreting B cells in the synovial fluid compartment. We hypothesised that the inflammatory environment could be crucial for sustained ACPA production and, indeed, found that cells present in synovial fluid can spontaneously generate a niche in culture in which antibody secreting cells from the SF secrete (auto)reactive antibodies for months. Further studies of these niches and the prolonged life span of antibody secreting cells could give relevant insight into therapeutic targets that shorten the life span of (autoreactive) B cells in the synovial compartment.

So far, our studies had demonstrated the presence of ACPA-expressing B cells and their secretory capacity, but the culture system used did not allow for detailed characterisation of ACPA-expressing B cells on the single cell level. As data on ACPA indicate that ACPA differ on the molecular level from other immunoglobulins, we hypothesised that the development of ACPA-expressing B cells could be different compared to most B cells and thereby result in differences in phenotypic and functional characteristics. Therefore, we focused in subsequent work on the development of an antigen specific staining for this autoreactive B cell population, as analysis of single cells could reveal detailed differences between ACPA-expressing B cells and the general
B cell population in individual patients. Difficulties hampering the successful identification of citrullinated antigen-specific B cells so far had been a lack of a positive control, a high background of the flow cytometry staining using control proteins/peptides, a suspected low frequency of these cells and the low affinity of their BCR. In chapter 4, we combined the availability of a sequence of a monoclonal ACPA[17] with a triple streptavidin tetramer staining approach to successfully identify citrullinated antigen-specific B cells. Using this technique, we found that most ACPA-expressing B cells in the periphery have a switched (mostly IgG) memory phenotype which could correspond to (resting) GC-derived memory B cells. We could also identify ACPA-expressing PB/PC, which could reflect a dynamic population, as well as some ACPA-expressing B cells with a seemingly naïve phenotype. The frequency of ACPA-expressing B cells correlated with ACPA serum titres and we could detect ACPA-expressing B cells in all isotypes, in-line with available data from studies of serum ACPA[18]. Our technique could be very valuable for further studies of ACPA-expressing B cells, as flow cytometry staining including markers of interest could be used to identify altered characteristics of ACPA B cells. Furthermore, FACS sorting experiments isolating ACPA-expressing B cells can allow the study of functional differences. Culturing ACPA-expressing B cells versus B cells with other specificities can be performed using different stimuli or (potential) therapeutics. Differences in response e.g. survival or Ig secretion could help to characterise functional differences. Both type of experiments help to understand the citrullinated antigen-specific immune response and could lead towards new therapeutic targets for ACPA (B cell) specific therapies. BCR repertoire analysis or mRNA sequencing could be used to further characterise the cells. This characterisation could lead to a better understanding of ACPA B cells, for instance, in terms of their development and/or activity. Furthermore, the CCP2 (cyclic citrullinated peptide) antigen in our tetramers could be exchanged for another peptide which is known to be recognized by (autoreactive) B cells. Performing this adjustment could give insight into other (autoreactive) B cells and their development and/or characteristics.

During B cell development, the (specificity of a) BCR is tested during several checkpoints for auto-reactivity. As autoreactive, ACPA-expressing B cells exist in the periphery of RA patients, we asked the question on how these cells form and/or escape selection checkpoints. During early B cell development, BCR make mostly use of kappa light chains (LC)[19, 20]. As B cells undergo rounds of B cell receptor editing, the chance of using a lambda LC increases. To investigate if formation of citrullinated antigen-specific B cells could be the result of B cells undergoing additional rounds of B cell receptor editing, potentially as a sign of selection pressure, we studied the kappa and lambda LC usage of citrullinated antigen-reactive BCR (chapter 5). BCR editing can occur in the bone marrow before ‘escaping’ negative selection or in germinal centre-like structures in RA patients, where the inflammatory environment makes it possible for B cells to alter their BCR. We studied the lambda LC usage of ACPA-IgG compared to total IgG showing an increased percentage of lambda LC usage in ACPA-IgG serum antibodies. We also observed an increased percentage of lambda LC
positive, ACPA-expressing CD27⁺ B cells. Of interest, we found that inhibition of interleukin (IL)-6 signalling in patients upon tocilizumab (aIL-6R) treatment reduced specifically the amount of ACPA-IgG using lambda LC within the first months. As the IL-6 signalling pathway is responsible for the (re-)expression of RAG enzymes required for BCR secondary rearrangements in synovial fluid/tissue[21], this indicates that interference with this pathway could prevent the development of part of lambda LC-positive ACPA resulting in lower ACPA titres and suggests that part of the ACPA-IgG,λ could have been formed upon secondary rearrangements in synovial fluid/tissue.

Recent data from murine studies show that the expression of CD28 on plasma cells promotes survival[22]. At the same time, inhibiting the interaction of CD28 with CD80/86 in RA patients treated with Abatacept (CTLA4-Ig) lowers ACPA serum titres and percentages of switched memory B cells[23]. Therefore, we set out to investigate if and to what extent CD28 is expressed by B cells of RA patients. We compared CD28 expression on peripheral B cell subsets of RA- and systemic lupus erythematosus (SLE)-patients, and of healthy controls (chapter 6). We could identify CD28-expressing B cells in comparable frequencies in the three groups. Interestingly, we found a higher frequency of CD28 expression on CD20⁺ B cells in SF obtained from RA patients and a trend towards a higher expression on PB/PC. Using the antigen-specific staining described in chapter 4, we detected CD28 expression, in similar frequencies, on ACPA-expressing plasmablasts. Whether these CD28-expressing, ACPA-expressing plasmablasts are the cells that survive in long-lived cultures, like in chapter 3, or in the circulation of RA patients is an interesting question and could be studied in future experiments, thereby providing insight into the survival of long-lived citrulline specific plasmacells in patients with RA.

Together, this thesis solves part of the puzzle concerning the characterisation of ACPA B cells and provides insights into the biology of the ACPA immune response. In the first part, we demonstrate the presence of ACPA-expressing B cells in different B cell subpopulations and estimate their frequency in peripheral blood as well as in synovial fluid. These data confirms presents of ACPA-expressing memory B cells and ACPA-secreting cells in peripheral blood. Presence of the latter are a sign of an active immune response. Analysis of lambda light chain usage hints towards a difference of the cells, giving insights into a maybe non-conventional development and thereby to a possible target for intervention. Single cell identification is possible since we succeed in the setup of an antigen-specific staining. This technique will be a very valuable tool for future studies allowing to dissect the characteristics and the study of the ACPA immune response. Together, this stepwise characterisation of ACPA B cells gives more insight into these autoreactive B cells. This thesis provides tools to further characterise these cells which will hopefully lead to better characterisation of B
cells and the underlying immune response. More understanding of autoreactive B cells could then provide better targets for therapeutic interventions. The latter could benefit patients with ACPA-positive RA or maybe even more general, patients with other unwanted autoreactive B cell responses.

References
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