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Chapter 5

Enhanced use of lambda light chains by citrullinated antigen-specific B cells: a sign of extensive receptor editing to avoid auto-reactivity?

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Abstract

Background: Despite considerable efforts in recent years to understand mechanisms of B cell tolerance, it remains unclear for most autoimmune diseases how autoreactive B cells arise and which (potentially defective) mechanisms govern their maturation. Secondary immunoglobulin light chain (LC) gene rearrangement in early B cell development represents an important mechanism of central tolerance aimed at eliminating autoreactive B cells. This mechanism, which depends on the activity of recombination activated genes (RAG) enzymes, has recently been shown to be operational also in antigen-activated mature B cells in the context of synovial inflammation, potentially to avoid auto-reactivity in the periphery.

Aim: To obtain insight into the characteristics and the development of human autoreactive B cells in the context of rheumatoid arthritis (RA), we studied the expression of functional kappa (κ) and lambda (λ) LC by citrullinated antigen-specific B cells and the LC usage of secreted anti-citrullinated protein antibodies (ACPA) in serum and synovial fluid of affected patients.

Methods: ACPA were isolated from serum and synovial fluid (SF) of ACPA-positive RA patients. Quantities of κ and λ LC and of total IgG of isolated ACPA or serum/SF depleted of ACPA were determined by ELISA. Peripheral blood from ACPA-positive RA patients was analysed by flow cytometry for the presence of kappa LC-expressing B cells in combination with specificity of the B cell receptor for citrullinated antigens.

Results: The κ-to-λ LC ratio of ACPA-IgG isolated from peripheral blood and SF was lower than the κ-to-λ LC ratio of ACPA-depleted total IgG. ACPA-IgG,λ was identified in a median percentage of 42% (IQR 33-54%) of total ACPA-IgG. ACPA were identified in a median frequency of 1 in 500 IgG,λ molecules (IQR 255-888), whereas the calculated frequency in the IgG,κ fraction was 1 in 560 (IQR 340-1200). Flow cytometry revealed reduced expression of functional kappa LC within the citrullinated antigen-specific B cell subset, corresponding to an enhanced frequency of lambda LC-positive B cells in this subset (median fraction lambda LC of total B cells: 0.42 (IQR 0.39-0.46); ACPA: 0.60 (IQR 0.55-0.79)). In patients before and three months after treatment with tocilizumab, we observed a decrease of ACPA-IgG in the total IgG,λ fraction but not in the total IgG,κ fraction.

Conclusions: The citrullinated antigen-specific B cell response more frequently generates lambda LC expressing B cells and secreted antibodies as compared to conventional immune responses. This could be the result of additional rounds of B cell receptor editing before entering the circulation or of secondary rearrangements in, for example, synovial tissue. This points to possible differences in the development of these autoreactive B cells and could help to unravel how autoreactive B cells arise.
Introduction

Anti-citrullinated protein antibodies (ACPA) are autoantibodies found in the majority of patients with rheumatoid arthritis (RA). Presence of ACPA associates with a severe disease phenotype[1] and ACPA can be found before clinical disease onset in some but not all patients[2]. ACPA are highly specific biomarkers for RA and various in vitro studies suggest that ACPA could promote in vivo inflammation by, for example, activating complement, mast cells, monocytes, neutrophils and osteoclasts[3-7].

Functional B cell receptors (BCR) consist of two heavy and two light chains (LC). LC contains a variable (V) and a joining (J) element and are encoded on two different loci: Ig kappa (κ) and lambda (λ). In total, the genes available allow for the expression of 320 different possible LC (40 Vκ x 5 Jκ + 30 Vλ x 4 Jλ[8-11]). Rearrangement of the first BCR during early B cell development results in most cases in a kappa LC-containing receptor[12, 13]. This BCR is often autoreactive. B cells expressing autoreactive receptors will be negatively selected before entering the periphery by either undergoing apoptosis, becoming anergic or by being allowed to modify their BCR by receptor editing. B cell receptor editing results in transcription of a different LC. This new LC can be further downstream on the same chromosome by deleting the locus of the previous LC, and when applicable in between loci, or the B cell can switch to the other kappa containing chromosome or switch to one of the lambda containing chromosomes. As a consequence, the chance of expressing a lambda LC increases with increased rounds of B cell receptor editing. Thus, a decreased κ-to-λ LC ratio could suggest that B cells underwent multiple rounds of B cell receptor editing before gaining their current specificity in an attempt to avoid auto-reactivity. However, the fate of autoreactive B cells is also determined by additional factors (such as the signalling strength through the BCR, affinity for the antigen, co-stimulatory factors, amongst others). Thus, every time a modified BCR is tested for auto-reactivity there is a chance that it escapes negative selection. This implies that a decreased κ-to-λ LC ratio could also be a sign that autoreactive B cells, upon altering their receptor (which could continue for several rounds), finally ‘escape’ and enter the periphery.

Several studies in mice and humans indicate that B cells can undergo secondary rearrangements in germinal centres and germinal centre like structures in synovium of RA patients[14-16]. To initiate Ig V-gene secondary rearrangements, recombination activating genes (RAG)-1 and RAG-2 need to be upregulated in B cells[17]. RAG-1 and RAG-2 binds recombination signal sequences (RSS) in between V domains and cleaves the DNA and holds it in place during receptor editing, finally resulting in a different BCR gene. Expression of RAG are confined to the developmental stage and is not found in peripheral blood B cells, but has been described in synovium of RA patients. Co-culture of fibroblast like synoviocytes (FLS) isolated from synovial tissue (ST) of RA patients and B cells result in the up-regulation of RAG genes and, as the B cell can thereby undergo additional rounds of B cell receptor editing, in increased usage of the lambda light chain. To obtain re-expression of RAG enzymes, the presence of BAFF and IL-6 is required[18]. Therefore inhibiting the IL-6
pathway by, for example, treating RA patients with the aIL-6R monoclonal antibody tocilizumab, could prevent the up-regulation of RAG genes in B cells in ST and thereby alter the κ-to-λ ratio in serum. As the ACPA immune response is an ongoing immune response, it could specifically result in less ACPA,λ after a few months of treatment.

The expression of κ- or λ LC has been described to be important in antibody specificities in different diseases. In systemic lupus erythematosus (SLE) it was found that λ LC using antibodies have a higher relative activity to hydrolyse human myelin basic protein (hMBP) compared to κ LC using antibodies[19]. In autoimmune pulmonary alveolar proteinosis (aPAP), the total concentration of GM-CSF autoantibodies does not correlate with disease severity but a lower κ-to-λ LC ratio does[20] suggesting that the LC usage might be associated with disease severity. Therefore a differential LC expression in ACPA could result in different effector functions of ACPA or be associated with a more severe disease phenotype. Although in RA the κ-to-λ LC ratio of total IgG has been compared to healthy donors previously[21], to our knowledge no study analysed the κ-to-λ LC ratio in RA for specific autoantibodies. As a second step, we questioned if ACPA-IgG, λ could have specific impact in RA patients which could be suggested if ACPA,λ level or κ-to-λ LC ratio would correlate with disease severity.

In the studies presented in this manuscript, we characterised the ACPA B cell response to obtain insight in how citrullinated antigen-specific B cells develop, using the κ-to-λ LC ratio in peripheral blood and synovial fluid as a proxy. We identified an increased λ LC usage in ACPA-IgG and by ACPA expressing B cells. Tocilizumab treatment, inhibiting IL6 signalling, resulted in a nonsignificant relative ACPA-IgG,λ decrease compared to total IgG,λ. Our data indicate that citrullinated antigen-specific B cells could be the result of additional rounds of B cell receptor editing which could indicate a difference in B cell development in these autoreactive B cells.

**Methods**

**Patients**

Peripheral blood, serum and synovial fluid samples were obtained on a cross-sectional basis from patients with ACPA-positive RA visiting the outpatient clinic of the Department of Rheumatology at Leiden University Medical Centre (LUMC), Leiden, The Netherlands. Patients fulfilled the 1987 criteria for RA at the time of diagnosis and gave written informed consent for sample acquisition. No selection based on specific treatments was performed. Peripheral blood samples were obtained from patients before their first tocilizumab treatment and three months thereafter. Permission for conduct of the study was obtained from the ethical review board of LUMC.

**ACPA purification using CCP2-coated beads**

ACPA and serum or synovial fluid (SF) depleted of ACPA were purified from serum and SF of patients with RA by antigen affinity chromatography using CCP2-coated beads, as previously described[22]. In short CCP2-coated beads
were generated by coupling biotinylated CCP2 (0.5 mg) to 5 ml of Pierce NeutrAvidin Plus UltraLink slurry resin (Thermo Scientific). After washing, the beads were loaded into a 96-well filter plate and 1:5 diluted serum or SF samples were incubated by shaking for 2h. The flow-through was obtained by centrifugation and one washing step, containing serum depleted of ACPA. ACPA were eluted from the resin by washing with 0.1M formic acid and neutralization with 2M Tris buffer.

**Measurement of IgG-kappa, IgG-lambda, total IgG and serum ACPA-IgG titres**

IgG light chain (LC) usage was assessed by coating ELISA plates with anti Igλ LC (clone JDC-12, BD Pharmingen) or anti Igκ LC (clone G20-193, BD pharmingen) monoclonal antibodies, followed by incubation with isolated ACPA or residual serum and then detected with polyclonal rabbit anti human IgG HRP (DAKO). The presence of total IgG was assessed by standard IgG ELISA (Bethyl Laboratories). To compare ELISA results, human reference serum (Bethyl Laboratories) was used as standard in all ELISA experiments. For analysis, arbitrary units of κ and λ were chosen relative to the amount of κ or λ in the aforementioned reference serum. Thereby the κ-to-λ ratio is compared to the ratio in this reference serum were the ratio 1.00 is equal to the ratio in the standard. ACPA-IgG titres in serum of tocilizumab treated patients were based on reactivity towards the CCP2-peptide. The CCP2-peptide was coupled to streptavidin-coated ELISA plates, followed by detection with polyclonal rabbit anti human IgG HRP (DAKO). Serum samples were tested at a 1:50 dilution or higher.

**Flow cytometry**

Streptavidin tetramer constructs were used to identify ACPA-expressing B cells as previously described[23]. PBMC were isolated from 40 ml of peripheral blood by Ficoll-Paque gradient centrifugation and stained with Fixable Violet (405nm) Dead Cell Stain Kit (Life technologies), CD3 Pacific Blue (clone UCHT1), CD14 Pacific Blue (clone M5E2), CD19 APC-Cy7 (clone SJ25C1), CD27 PE-Cy7 (clone M-T271), IgG-BV510 (clone G18-14), IgA-FITC (DAKO), IgM-PerCP Cy5.5 (clone G20-127), IgD-PE-CF594 (clone IA6-2), Igκ light chain-Alexa Fluor 700 (clone G20-193, (all except IgA) BD Biosciences), CCP2-coupled APC- and BV605-tetramers, and with a PE-labelled arginine control (CArgP2) tetramer.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 6.02. Correlations were assessed as non-parametric paired correlations. p Values <0.05 were considered significant.
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Results

κ-to-λ light chain ratio of ACPA-IgG is decreased compared to total IgG

To obtain insight into the citrullinated antigen-specific immune response we studied if the κ and λ light chain (LC) usage of ACPA deviates from the κ and λ LC usage of total IgG. To this end, we isolated ACPA from serum and measured κ and λ IgG and calculated the κ-to-λ LC IgG ratio of isolated ACPA and residual serum (IgG depleted of ACPA). As was described previously[21], we confirmed that RA patients have a normal[21, 24] IgG 2-to-1 κ-to-λ ratio (figure 5.1A). Interestingly, we found that the κ-to-λ LC ratio of ACPA-IgG is significantly decreased to 0.76 (IQR 0.53-1.00) corresponding to a median of 1.4-to-1 κ-to-λ LC (58% κ and 42% λ). No correlation was observed between this ratio and ACPA-IgG serum tireds (data not shown).
As it has been described that fibroblast-like synoviocytes from synovial tissue (ST) can provide the signals required to up-regulate RAG genes in B cells and thereby can promote κ-to-λ LC switching in vitro[18], we questioned if the relative frequency of ACPA-IgG, λ LC in synovial fluid would be increased. Again, we observed a decreased κ-to-λ ratio in ACPA-IgG but did not find a different ratio compared to serum (figure 5.1B). This was also the case if only paired samples were analysed (data not shown).

Calculating the frequency of ACPA-IgG, λ in IgG, λ or ACPA-IgG, κ in IgG, κ could give additional insights into our data. This to see if the changes in ratio are more due to a decrease in kappa LC or an increase in lambda LC-using ACPA when analysing at different time points or when correlating kappa or lambda LC-using ACPA to disease activity. The identified frequency was 1 in 500 IgG, λ as ACPA (IQR 255-888) and 1 in 560 IgG, κ as ACPA (IQR 340-1200) (figure 5.2). We did not find a correlation between the amount of ACPA-IgG, λ, ACPA-IgG, κ or κ-to-λ LC ratio and disease severity (data not shown).

**Figure 5.2: Median frequency of ACPA for lambda and kappa LC IgG’s. Left a median of 1 in 500 IgG, λ was identified as ACPA (IQR 255-888) and right a median of 1 in 560 IgG, κ was identified as ACPA (IQR 340-1200). Data was assessed as non-parametric paired samples.**

**Distribution of λ and κ light chain usage in ACPA-expressing B cells**
Antibodies in serum are secreted by antibody secreting cells. Although we have previously identified such cells for the citrullinated antigen-specific repertoire in peripheral blood[25], it is unknown whether ACPA-expressing cells in the periphery also show a shifted expression of λ LC. To answer this question, we combined our previously described antigen specific staining[23] with a staining for LC. Since λ LC was not but κ LC was available coupled to a fluorochrome which could be implemented in the previous described antigen specific staining we used κ LC as a proxy. In the left part of figure 5.3A we presented a different
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Distribution of κ expressing B cells in the citrullinated antigen-specific as compared to the total B cell population. To exclude the possibility that this is driven by a higher percentage of CD27⁺ ACPA-expressing B cells, we also analysed the data by only evaluating CD27⁺ B cells (right part figure 5.3A). This identified a median of 70% of CD27⁺ ACPA-expressing B cells expressing λ LC. Both CD27⁺ IgG⁺ and CD27⁺ IgA⁺ ACPA-expressing B cells showed this preference for λ LC expression, with medians of 73% and 90%, respectively (figure 5.3B).

Effect of tocilizumab treatment on Serum ACPA-IgG and IgG

Given that IL-6 is required for the re-expression of RAG enzymes when B cells undergo secondary rearrangements, we hypothesised that inhibition of IL-6 signalling during treatment with tocilizumab could result in less newly expressed ACPA-IgG carrying λ LC. Our previous data demonstrated that the citrullinated antigen-specific immune response is continuously generating new ACPA B cells, making it likely that a decline in ACPA-IgG,λ could be observed in tocilizumab-treated patients. Therefore, we calculated the ratio of ACPA-IgG prior to compared to three months after first tocilizumab treatment in six patients (figure 5.4A). Although the changes observed were not significant, most patients showed a trend towards lower ratios for ACPA-IgG while the same ratios tended to increase for total IgG.
To test if tocilizumab treatment specifically inhibited the active immune response resulting in less formation of ACPA, 5 to total IgG titres, we isolated ACPA from these sera and measured ACPA-IgG and total IgG and calculated as in figure 5.2 the frequency of IgG/ACPA (figure 5.4B). Although no significant differences were observed in these nine patients, the median of the IgG/ACPA lambda ratio before treatment was lower than after three months indicating a relative decrease of ACPA-IgG, 5 to total IgG, k in serum, whereas the relative ACPA-IgG, k to total IgG, k remained unchanged.

**Discussion**

In this manuscript, we describe that ACPA-IgG isolated from peripheral blood of ACPA positive RA patients contains a higher frequency of lambda LC compared to total IgG of the same patients. In SF, the ACPA-IgG lambda LC usage was also increased and similar to the frequency found in peripheral blood. Although a decreased k-to- 5 LC ratio in other diseases has been linked to disease severity, we could not find such a correlation for ACPA-IgG, nor did we find a correlation with ACPA-IgG serum titres. We also show that ACPA-expressing B cells more frequently express 5 LC as compared to non citrullinated antigen-specific B cells. In patients treated with tocilizumab, we observed a trend towards decreased ACPA-IgG titres and increased IgG titres within three months of treatment. By analysing lambda and kappa LC IgG separately, we observed a relative decrease in ACPA-IgG, 5 compared to ACPA-IgG, k. These data suggest that tocilizumab treatment alters the IgG/ACPA-IgG lambda LC usage, and that part of ACPA-IgG that express lambda LC could be generated upon secondary rearrangements since this is (partially) blocked by treatment.
We observed that serum ACPA-IgG molecules more frequently contain lambda LC compared to total IgG. Even more interesting, not only the κ-to-λ LC distribution of serum antibodies but also the λ LC usage on ACPA-expressing switched memory B cells was higher compared to the total switched memory B cell population. This indicates that citrullinated antigen-specific B cells underwent more rounds of B cell receptor editing. In terms of regular B cell biology this could be the result of two different processes. First it could be a failure in the checkpoint deleting autoantibody-expressing B cells before they enter the periphery, where the autoreactive citrullinated antigen recognizing B cells do not go into apoptosis or undergo additional rounds of B cell receptor editing. Alternatively, secondary rearrangements and tolerance breakdown occurs in ST of RA patients and thereby results in autoreactive antibodies with an increased lambda usage.

As secondary rearrangements in ST are known to occur and lead to enhanced lambda usage[18] and as it was shown that B cells in ST are clonally related[15], we questioned whether lambda LC usage of ACPA-IgG in SF is increased. Although we found a similar difference comparing the κ-to-λ LC ratio of ACPA-IgG to total IgG in synovial fluid compared to peripheral blood, we did not observe more increased frequency of lambda LC usage in the SF. It could be possible that diffusion of antibodies secreted in SF or peripheral blood to the other compartment is quite quick, therefore no differences can be measured. Therefore, in future experiments antigen specific kappa LC staining on synovial fluid B cells or synovial tissue could give more insight into the question whether the higher ACPA,λ frequency could be due to secondary rearrangements in ST of RA patients.

Although in autoimmune pulmonary alveolar proteinosis (aPAP)[20] it was found that a decreased κ-to-λ LC ratio correlates with disease severity, for ACPA we did not observe a correlation. This with the note that ACPA is polyclonal and we did not look into fine specificities. Furthermore it would be interesting to see if the ACPA κ-to-λ LC ratio is already decreased in individuals before disease onset or decreases further depending on disease duration.

To see if tocilizumab can inhibit the development ACPA-IgG,λ B cells we studied the ACPA-IgG and total IgG in serum of patients before and three months after their first tocilizumab treatment. We observed a trend towards less ACPA-IgG,λ compared to total IgG,λ upon treatment. ACPA reflect an active immune response and we have demonstrated the presence of citrullinated antigen-specific plasmablasts/-cells in peripheral blood previously[25]. As the general life span of plasmablasts is short, and formation of new ACPA-IgG,λ secreting cells is, conceivably, inhibited by blocking IL-6 signalling, these data could suggest that short lived ACPA secreting plasmablasts died and less ACPA-IgG,λ expressing B cells were newly generated in these patients. This results in decreasing ACPA-IgG,λ serum titres as IgG gets cleared over time and less
new ACPA-IgG,λ will be secreted. Since no differences were observed in these patients for ACPA-IgG,κ our data indicate that generation of ACPA-IgG,κ secreting B cells is not dependent on IL-6 signalling. It would be interesting to see how IL-6 blockade is modulating the ACPA immune response in patients. Further studies should be done to investigate if IL-6 blockade is indeed directly involved in inhibiting secondary rearrangements resulting in less ACPA,λ and if this effect is long term and ACPA,λ specific.

In conclusion, ACPA-IgG more frequently contain lambda LC. Turning this observation around: altering the LC of an antibody increases the chance that a lambda LC is used and statistically also increases the cumulative chance that an autoreactive cell has escaped negative selection although the specificity is (still) autoreactive. If these autoreactive B cells are specific for citrullinated antigens this would result in an increased λ LC usage of citrullinated antigen-specific B cells. This citrullinated antigen specificity can be gained early in B cell development in the bone marrow but could develop upon secondary rearrangements in local germinal centre like structures in RA patients. In this manuscript, we gained more insight into the biology of citrullinated antigen-specific B cells, demonstrating that these cells show an increased lambda LC usage. This could indicate active B cell receptor editing which could be related to their existence in the periphery as a result of defective negative selection. Further studies are needed to better elucidate the development of these cells.

References
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