Chapter 2

Anti-citrullinated protein antibodies have a low avidity compared to antibodies against recall-antigens.

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ABSTRACT

Objectives

Anti-Citrullinated Protein Antibodies (ACPA) are highly specific for Rheumatoid Arthritis (RA) and have been implicated in disease pathogenesis. Recent ongoing evidence indicates that the ACPA-response broadens before precipitation of full-blown RA as indicated by a more extensive isotype usage and an increased citrullinated epitope recognition profile. Nonetheless, the evolution of the ACPA-response is still poorly understood and might intrinsically differ from the protective responses against pathogens.

Methods

We analyzed the avidity and the avidity maturation of ACPA in relation to the avidity of antibodies against recall antigens.

Results

The avidity of ACPA was significantly lower compared to the avidity of antibodies to the recall antigens Tetanus Toxoid (TT) and Diptheria Toxoid (DT). Moreover, ACPA did not show avidity maturation during longitudinal follow-up and ACPA avidity was relatively low also in patients that displayed extensive isotype switching.

Conclusions

These observations indicate that the natural evolution of ACPA differs from the development of antibodies against recall antigens. Moreover, these data indicate that ACPA avidity maturation and isotype-switching are disconnected whereby extensive isotype switching occurs in the setting of restricted avidity maturation. Intrinsic differences between RA specific autoantibody system and protective antibody responses against pathogens could be of relevance for designing novel B cell targeted therapies for RA.

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic autoimmune disease that mainly affects the joints. The identification of anti-citrullinated protein antibodies (ACPA) represents an important breakthrough in the field of RA [1,2]. ACPA recognize post-translationally modified proteins, in which arginines have been modified into citrullinated residues by peptidylarginine deiminase (PAD) enzyme during inflammation. Therefore it is hypothesized that citrullination of proteins in the joint may create epitopes that can serve as targets of ACPA, ultimately leading to inflammation and arthritis. Indeed several observations implicate ACPA in disease pathogenesis as it has been shown that the presence of ACPA predicts the emergence and outcome of RA [3-5]. Moreover, ACPA have been implicated in disease pathogenesis by the observations that ACPA can induce and aggravate arthritis in mice [6,7] and can activate human immune effector mechanisms, such as triggering of cellular Fc receptors [8] and activation of the complement system [9].

During a B cell response, isotype switching and affinity maturation typically occurs in the germinal center. Following somatic hypermutation, different B cell clones will compete for antigen on follicular dendritic cells (FDC). The B cells expressing surface immunoglobulins with a higher avidity will acquire the signals necessary for survival and proliferation. As a result, the total avidity of the immune response increases because low avidity B cells will not be stimulated and will eventually disappear from the population.

By definition, antibody affinity is the strength of interaction between a single antigen binding sites and soluble monovalent antigens in solutions. However, in reality antibodies are multivalent and contain 2 (IgG) to 10 (IgM) antigen binding sites. In addition, also the antigens are often multivalent and/or nonsoluble. Therefore antibody avidity which is defined as the overall binding strength of polyclonal antibodies to a multivalent antigen provides a better measure for the strength of antibody responses.

Extensive information has been obtained regarding the avidity maturation of antibody responses against recall antigens, mostly following vaccination [10-12]. Interestingly, in the mouse it was recently described that B-cells producing arthritogenic antibodies are relatively short lived plasmablasts that are different from the long lived plasma cells typically producing protective antibodies [13].

Given the implicated role of ACPA in RA, we have now investigated ACPA-avidity and avidity maturation in arthritis patients in relation to avidity of recall antigens.

MATERIALS AND METHODS

Patients

Sera of 92, ACPA positive, patients with early arthritis were selected from the early arthritis clinic (EAC), an inception cohort of recent onset arthritis previous described [14]. Sera for longitudinal analysis were selected from available samples at baseline, 1 year and 5 year follow-up. Characteristic of patients in our cohort were as follows: 65.2% are female, age at inclusion is 51.7(17.0-82.3) years, disease duration is 7.71(0.2-36.13) months, 80.4% are RF positive and 66.3% have erosion at baseline. These individuals were not vaccinated with TT and DT for the purpose of this study. We also studied ACPA avidity at baseline during active disease and at remission in 5 RA patients with complete drug free remission. From 15 randomly selected RA patients we analyzed paired serum and synovial fluid samples. Disease duration of these patients is 8.27 (1-21) years. The collection and use of patient samples was approved by the local medical ethics committee in compliance with the Helsinki declaration.

Avidity assays for ACPA and recall antigens

To determine the avidity of ACPA IgG and IgG antibodies against recall antigens we used elution enzyme-linked immunosorbent assay (ELISA) assays [15,16].

For ACPA the appropriate serum dilution was first determined by performing a titration using a CCP2 ELISA (Immunoscan RA Mark 2, Euro-Diagnostica, Arnhem, The Netherlands) with minor modifications, now using 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic-acid substrate. The serum dilution at which the response was 50% of maximum was considered 'optimal' and a minimal dilution we used is 1:25. In addition, after developing the plate for 1 hour the absorbance at 415 nm had to be between 0.5-2.0 to allow optimal detection (figure 1A).

To determine the avidity of the anti-CCP2 antibodies, plates were incubated with the appropriate serum dilutions in PBS-Tween 1% BSA (PTB), for 1 hour at 37^{o}C. After washing, the wells were incubated with increasing concentrations of the chaotropic agent sodiumthiocyanate: NaSCN at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4 and 5M, for 15 minutes at RT. The wells were washed and bound antibodies were detected using HRP-labeled goat-anti-human IgG (DAKO, Denmark). The amount of antibody bound to the plate without elution and the amount that resisted elution by NaSCN were determined relative to a standard curve.

No difference in avidity was detected where ACPA avidities were measured on commercial plates or on in house plates coated with CCP2 peptide (data not shown).

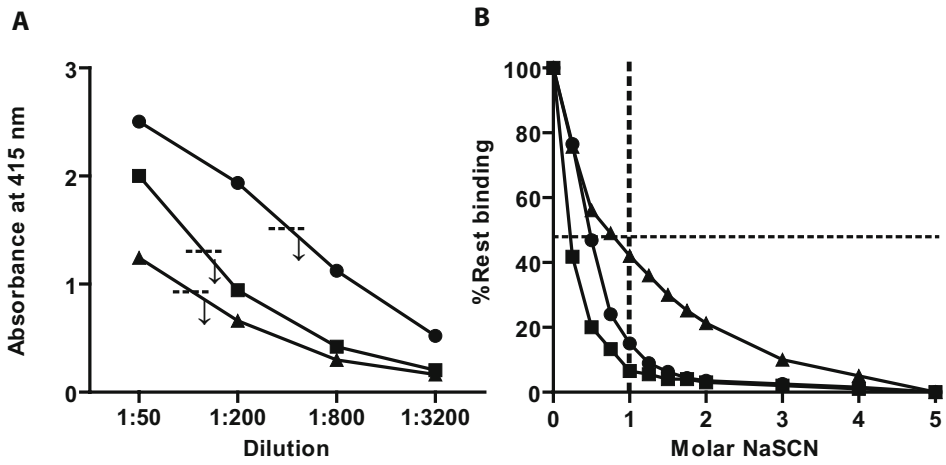


Figure 1. Characteristics of the avidity assay.
A. The appropriate serum dilution for the avidity assay was determined by performing a dose response study. The serum dilution was chosen as to obtain a 50% of maximal binding (arrows). Three representative samples for anti-CCP2 reactivity are presented.
B. The relative Avidity Index (AI) is defined as the ratio of the amount of residual antibodies bound to the coated antigen after NaSCN elution (indicated by the vertical dotted line) relative to the amount of binding antibodies in the absence of NaSCN, expressed as percentage. The horizontal line indicates the 50% elution profile which is used to calculate the concentration NaSCN that is necessary to elute 50% of the antibodies. Three representative samples for anti-CCP2 are presented.

The "relative avidity index" (AI) was calculated [17]. The AI is defined as the ratio of the amount of residual antibodies bound to the coated antigen after NaSCN (1M) elution to the amount of binding antibodies in the absence of NaSCN, expressed as percentage (figure 1B).

$$AI = \frac{\text{remaining antibodies at 1M NaSCN (AU/ml)}}{\text{binding antibodies at 0M NaSCN (AU/ml)}} \times 100$$

To determine the avidity and levels of antibodies against citrullinated human fibrinogen (cit Fib) we used a plate bound assay as described before [18] with minor modifications.

To determine the avidity of antibodies against modified citrullinated vimentin (anti MCV) we used a commercial ELISA (ORGENTEC Diagnostica GmbH, Mainz, Germany).

To determine the avidity of anti-TT IgG and anti-DT IgG we used an in-house ELISA [19,20]. In short, plates were coated with 100 ul/well of TT (1.5 Lf/ml in 0.05 M carbonate buffer, pH 9.6) or DT (0.75 Lf/ml in carbonate buffer, pH 9.6) both from RIVM (Bilthoven, The Netherlands).

Avidity of anti cit Fib, anti MCV, anti-TT IgG and anti-DT IgG antibodies were determined as for anti-CCP2 antibodies.

In all cases, conditions used for each of the assays were optimized to allow maximal antibody binding. The buffers used for coating antigens are washed away before avidity measurement and thus will not impact on the avidity measurement. After the antibodies have bound to their different antigens, the plates are washed with the same buffer and eluted with elution buffer, and subjected to a similar detection of the residual antibody binding to the plate. In this way, this assay allows the comparison of avidities of antibodies binding to different antigens [12].

Since the presence of Rheumatoid Factor (RF) could potentially influence our observations, we analyzed the occurrence of RF in the low and high avidity groups. No correlation between RF positive status and ACPA avidity was observed (data not shown). In addition, we measured avidity of RF IgM and IgG in three patients and observed also for RF a low avidity compared to avidity of anti-TT and anti-DT (data not shown).

Isotype measurements were performed as described [21].

Statistical analysis

Differences between groups were analyzed with the Mann-Whitney test or ANOVA, and correlation was determined by the Spearman's correlation coefficient with GraphPad Prism 4.0 software (GraphPad Inc, San Diego, CA, USA) or SPSS for Windows (release 16.0, SPSS, Chicago, IL, USA). In all tests, $P < 0.05$ was considered significant.

RESULTS

The avidity of ACPA is low compared to the avidity of antibodies against recall-antigens

As the avidity of antibodies towards antigens can have important implications for the biological effects mediated, we wished to determine the avidity of the ACPA response in RA patients and compared the avidity of ACPA IgG to the avidity of IgG against recall antigens.

In an initial group of 8 patients we analyzed the avidity of antibodies directed against three citrullinated antigens; the CCP2 peptide and two citrullinated proteins; cit-Fib and MCV, as well as the avidity of antibodies against the T cell dependent - recall protein antigens TT and DT. As shown in Figure 2A we observed, within the same patients, a low avidity for antibodies directed against all three

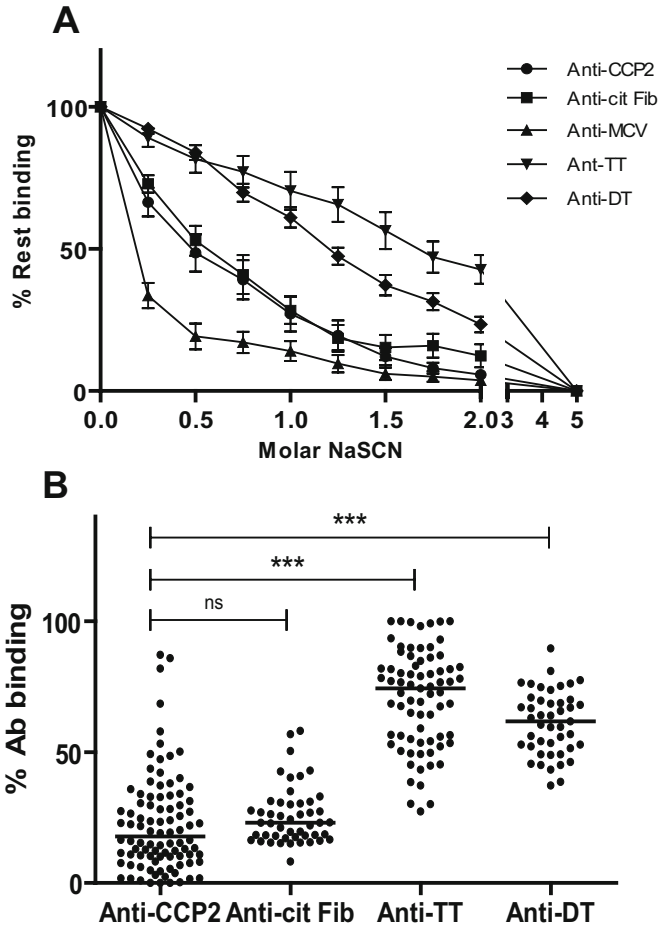


Figure 2. The avidity of ACPA IgG is lower than the avidity of IgG against recall antigens.
A. Avidity of ACPA IgG, anti-CCP2, anti-cit Fib and anti-MCV were compared to the avidity of IgG against recall antigens TT and DT in 8 patients. The data of the elution profiles are presented as mean +/- SEM.
B. Avidity of ACPA IgG, anti-CCP2 and anti-cit Fib, were compared to the avidity of IgG against the recall antigens TT and DT in 67 patients and is expressed as relative AI. The avidity of ACPA IgG is significantly lower than the avidity of IgG to recall antigens (P-value < 0.0001).

different citrullinated antigens and a high avidity for antibodies against recall antigens TT and DT. To study this phenomenon in a larger cohort we focused on CCP2 and cit-Fib versus TT and DT. As shown in Figure 2B, in the cohort as a whole, the avidity of anti-CCP2 antibodies is generally low (median 19%), with most patients displaying an avidity with an AI lower than 40% (Figure 2B). Next we also analyzed the avidity of antibodies directed against cit-Fib, an entire citrullinated protein, similar results were obtained. In sharp contrast, the avid-

ity of antibodies against the recall antigens TT and DT (medians 74% and 61%, respectively) are of high avidity (Figure 2B), with most individuals displaying an AI of more than 40%. This is significantly higher than the avidity of ACPA ($P<0.0001$). Thus our data show that these ACPA are of low avidity as compared to antibodies against recall antigens.

ACPA IgG avidity in sera represents overall avidity of ACPA

To control for the possibility that the overall avidity of ACPA, as detected in serum, is influenced by retention of high avidity ACPA in the inflamed joint, we next analyzed ACPA avidity of patients during active disease and compared it to the avidity of ACPA of the same patients during complete drug free remission (N=5). We did not observe an increase in the overall avidity of ACPA indicating that ACPA avidity is not influenced by disease activity and that there is no evidence for a retention of high avidity antibodies in the inflamed joint (figure 3A). Likewise, we have analyzed if there is a difference in the avidity of ACPA IgG between patients that are only IgG positive to patients that are also positive for IgA and / or IgM ACPA. There was no difference in the IgG ACPA avidity depending on the presence of either IgM or IgA ACPA (data not shown). Furthermore we purified IgG from serum that was positive for IgG, IgA and IgM ACPA and compared the ACPA IgG avidity from serum to that of the purified IgG and observed a similar avidity. Together these data indicate that the other isotypes had no impact on the avidity measurement of ACPA IgG (data not shown).

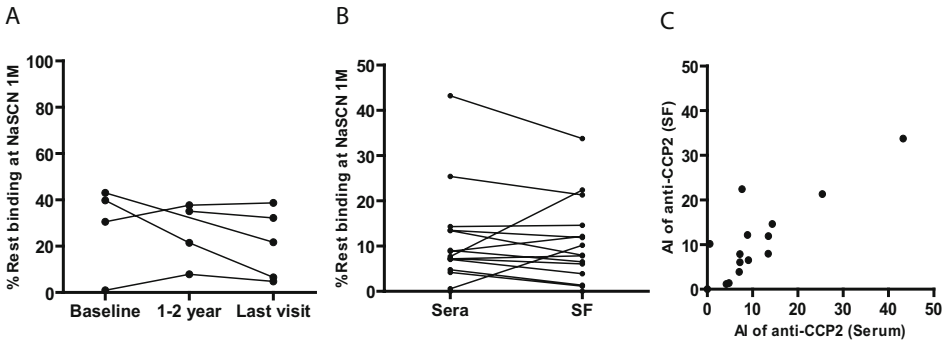


Figure 3. Avidity of ACPA IgG in serum is not influenced by disease activity and is similar to ACPA IgG in synovial fluid.

A. Comparison of the avidity of ACPA IgG in serum measured in RA patients during active disease at baseline to complete absence of disease activity at drug free remission. The avidity of ACPA measured in serum is not influenced by disease activity.

B. Avidity of ACPA in sera compared to avidity of ACPA in paired samples of synovial fluid.

C. Correlation between avidity of anti-CCP2 in sera (x-axis) and in paired synovial fluid (y-axis).

We also compared the avidity of anti-CCP2 in synovial fluid to the avidity of anti-CCP2 in paired sera. Avidity of anti-CCP2 in synovial fluid was comparable to avidity of anti-CCP2 in sera (Fig.3B,C). Together, these data indicate that the avidity of ACPA as measured in serum does provide a good representation of the overall ACPA avidity.

The diversity of avidities of ACPA differs from the avidity diversity of recall antigens.

So far we have depicted the avidity as avidity index (AI), a measure of the percentage of antibody still bound after elution with 1M NaSCN. However, since it

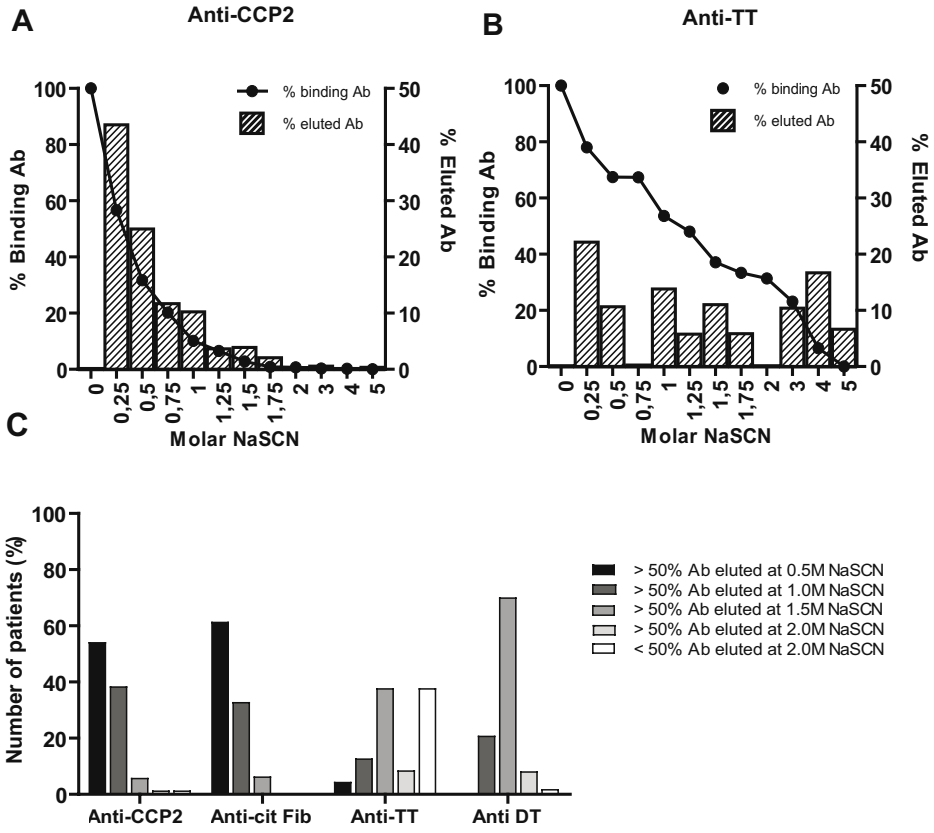


Figure 4. Different elution profiles between ACPA and IgG against recall antigens.
Different patterns of elution between anti-CCP2 IgG (A), and anti-TT IgG (B) following NaSCN elution. Percentage of antibody that was eluted after each consecutive step of NaSCN elution is expressed in bars and the percentage of antibody still bound after NaSCN elution is presented as a linear graph. The elution profiles of anti-CCP2 IgG and anti-TT IgG of one representative patient are shown. C) The proportion of patients that have different elution profiles for the indicated antibody response.

is possible that elution profiles differ while having a similar AI, we also analyzed the elution profiles. Different antibody elution patterns for ACPA in comparison to IgG against recall antigens within one subject were observed (figure 4). For example, as presented in figure 4, the avidity of anti-CCP2 IgG present in the sample analyzed displayed a rather homogenous response, with over 80% of the antibodies eluting in the first 3 elution steps (Figure 4A). In contrast, a wide distribution of avidities is observed for IgG against TT (Figure 4B). Interestingly, the antibody response to recall antigens displays a wider distribution of avidity, whereas the avidity distribution of the ACPA response is, in general, narrow (Figure 4C). The absence of heterogeneity within the avidity-profile of ACPA, further indicates limited avidity maturation of the ACPA response.

Dynamics of ACPA IgG avidity

To evaluate whether there is affinity maturation of the ACPA IgG response over time following onset of arthritis, we next determined the avidity of ACPA in a longitudinal fashion, by comparing samples from the same patients at baseline and at 1 and 5 years follow up. We did not observe a correlation between ACPA avidity and baseline clinical characteristic such as age of onset, sex, DAS or CRP (data not shown). We observed no increase in ACPA IgG avidity over time in the patients analyzed (Figure 5A). As expected, the result for avidity measurements of anti-cit Fib antibodies in time confirms this observation (Figure 5B). In addition we did not observe a change in the high avidity (>2M) population of ACPA during follow up of patients (data not shown). These data indicate that during disease

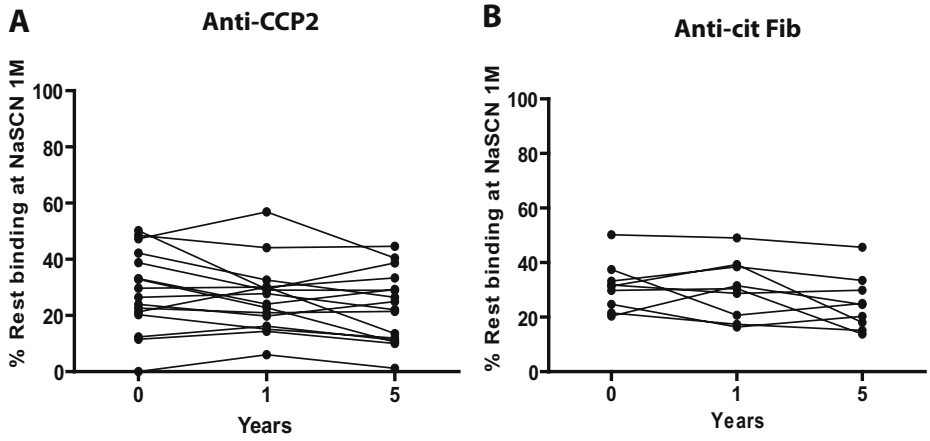


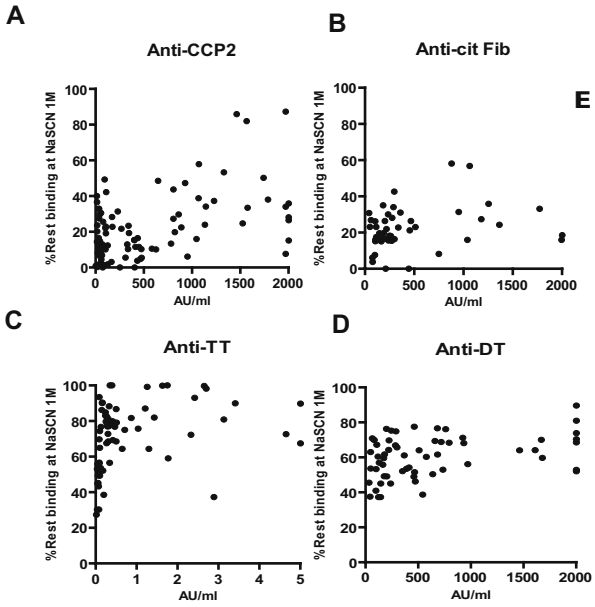
Figure 5. No avidity maturation of ACPA IgG during 5 year follow-up.
The avidity of ACPA in serial samples at baseline, 1 and 5 years follow-up were analyzed. Anti-CCP2 avidity was analyzed in 16 RA patients (A) and anti-cit Fib avidity was analyzed in 9 RA patients (B). The ACPA response did not show extensive avidity maturation.

progression the ACPA response does not undergo extensive avidity maturation. However, these data do not exclude limited avidity maturation before disease onset.

ACPA avidity maturation, titers and isotypes.

We determined the relation between antibody levels and avidity of both ACPA and IgG against recall antigens (Figure 6A-D). The data indicate that the avidity of IgG against recall antigens can be high even if the titer is low, confirming previous results. More importantly, however, patients with a high titer of ACPA IgG still display only a relatively low avidity. This contrasts to antibodies against recall antigens which have a high avidity in case of a high titer.

Likewise, the isotype usage of ACPA was assessed in relation to the avidity of ACPA IgG. Unlike the distribution of the isotype usage of anti-TT antibodies, which is predominated by IgG1 (data not shown)[19]. We analyzed isotype usage in 8 patients with the lowest ACPA avidity and observed that there is broad



E Broad ACPA isotype usage in low avidity ACPA

Low	IgG	IgM	IgA	IgG1	IgG2	IgG3	IgG4
I	X	X	X	X	X	X	X
II	X		X	X	X	X	X
III	X		X	X	X	X	X
IV	X			X		X	X
V	X			X	X		X
VI	X	X		X	X		X
VII	X			X			X
VIII	X		X	X	X		X

Figure 6. ACPA avidity maturation, titers and isotypes

Correlation between the levels and avidity of ACPA IgG; anti-CCP2 (A) and anti-cit Fib (B), and IgG against recall antigens; anti-TT (C) and anti-DT (D). (E) Broad ACPA isotype usage also in patients with low avidity of ACPA IgG. Shown are the 8 patients with the lowest avidity (numbered I – VIII) with their use of isotypes in the ACPA response (X indicates positivity for an isotype).

usage of isotypes even in these patients (Figure 6E). Together these data reveal poor avidity maturation of ACPA IgG, despite high antibody titers and extensive isotype switching.

Discussion

In this study the ACPA response is generally of a much lower avidity than the recall responses and in our limited longitudinal study we did not obtained evidence for avidity maturation during the course of established disease. These data indicate that the regulation of the RA-specific autoimmune response against citrullinated antigens differs from the regulation of recall-responses.

Currently, only limited information is available on the endogenous citrullinated antigens recognized by ACPA. Therefore, we have used three different antigens for the determination of ACPA-avidity; the CCP2 peptide and two citrullinated proteins. The CCP2 ELISA is the most commonly used for the detection of ACPA [22], whereas the proteins cit-Fib and MCV have been shown to be present in the sera, synovial fluid and synovium of RA-patients [23,24] and are recognized by ACPA of most RA-patients [25]. We made similar observations for all three systems analyzed, providing internal confirmation of the results.

To exclude the possibility that the observed low avidity of ACPA detected in serum was a reflection of preferential retention of high avidity antibodies in the inflamed joint we analyzed ACPA avidity during active disease and complete drug free remission, where all clinical activity was absent. These studies indicate that there is no change in the avidity of ACPA detected in serum comparing active disease to remission. As we also did not observe a difference in ACPA avidity in paired samples of SF and serum, we consider it unlikely that the preferential retention of high avidity antibodies in the joint due to the presence of citrullinated epitopes explains the low avidity of ACPA, although this possibility can not formally be ruled out.

The difference in the ACPA response as compared to 'conventional' B cell responses studied (i.e. B-cell responses against recall antigens) might be due to the nature of the antigen where citrullinated antigens are likely to be presented constantly in the body at multiple site, where recall antigens are presented for a short amount of time at a localized site. This would lead in a relative abundance of citrullinated antigens in the body and, as a consequence, the absence of competition for antigens by different B-cell clones in the germinal center as to be expected for recall antigens, absence of affinity maturation and hence low-affinity antibodies. Unlike isotype switching, avidity maturation not only critically depends on the presence of antigen, but most likely also on proper amounts of antigen in the

germinal centers [26]. This will result in an antibody response that does undergo isotype switching but does not display avidity maturation as we observed both at baseline and at follow-up. We favor this explanation over other possibilities such as, for example, lack of adequate T cell help on a limited amount of time passed after antigen exposure. The ACPA response is characterized by extensive isotype switching, including IgA and IgE [18,21,27]. The generation of these isotypes appears T-helper cell-dependent as patients suffering from hyper IgM syndrome (caused by a gene defect in CD40Ligand) do not develop IgA responses [28,29]. Moreover, the HLA alleles predisposing to RA, only predispose to ACPA-positive RA and not to ACPA-negative disease [30] also indicates the involvement of CD4+ T helper cells in the formation of ACPA. Likewise, avidity maturation of antibody responses against recall antigen takes place within weeks, whereas the disease duration of most RA patients is much longer.

Our data indicate that ACPA producing B cells behave differently as compared to 'conventional' B cells. Recently it was shown in mice that rituximab specifically depletes B cells producing the autoantibodies, while sparing the 'conventional' plasma cells producing the protective antibodies [13]. Therefore therapies targeting the crucial biological mechanisms underlying 'conventional' B cell responses may not work similarly on ACPA producing B cells. In this respect, a possible interaction that could be of relevance is a therapy targeting the survival factors B-cells compete for during the germinal center reaction [31].

In conclusions, our data indicate that the ACPA response is different from antibody responses against recall protein antigens. The ACPA response can be of high titer, can use all isotypes yet is of low avidity that contrasts to antibodies against recall antigens that are of high avidity. These and possibly other differences between ACPA and recall responses are likely reflecting differences in the underlying B cells response. Understanding these differences may be of relevance for the design of novel B cell targeted therapies in RA.

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