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# B cell depletion with rituximab in patients with rheumatoid arthritis: Multiplex bead array reveals the kinetics of IgG and IgA antibodies to citrullinated antigens

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ABSTRACT

The serology of patients with Rheumatoid arthritis (RA) is characterized by persistently raised levels of autoantibodies: Rheumatoid Factors (RhF) against Fc of IgG, and to citrullinated (Cit) protein/peptide sequences: ACPA, recognizing multiple Cit-sequences. B cell depletion therapy based on rituximab delivers good clinical responses in RA patients, particularly in the seropositive group, with responses sometimes lasting beyond the phase of B cell reconstitution. In general, ACPA levels fall following rituximab, but fluctuations with respect to predicting relapse have proved disappointing. In order to identify possible immunodominant specificities within either IgG- or IgA-ACPA we used a Multiplex bead-based array consisting of 30 Cit-peptides/proteins and 22 corresponding native sequences. The kinetics of the serum ACPA response to individual specificities was measured at key points (Baseline, B cell depletion phase, Relapse) within an initial cycle of rituximab therapy in 16 consecutive patients with severe, active RA. All had achieved significant decreases in Disease Activity Scores-28 and maintained B cell depletion in the peripheral blood (<5 CD19+cells/μl) for at least 3 months. At Baseline, mean fluorescence intensity shown by individual IgG- and IgA-ACPA were strongly correlated ($R^2 = 0.75$; $p < 0.0001$) but IgA-ACPA were approximately 10-fold lower. Data were Z-normalised in order to compare serial results and antibody classes. At Baseline, a total of 68 IgG- and 51 IgA-ACPA had Z-scores $\geq 1$ (above population mean) were identified, with at least one Cit-antigen identified in each serum. ACPA to individual specificities subsequently fluctuated with 3 different patterns. Most 51/68 (75%) IgG- and 48/51 IgA-ACPA (94%) fell between Baseline and Depletion, of which 57% IgG- and 65% IgA-ACPA rebounded pre-Relapse. Interestingly, 17/68 IgG-ACPA (25%) and some IgA-ACPA (3/51; 6%) transiently increased from Baseline, subsequently falling pre-Relapse. Individual responses to particular Cit-epitopes were not linked to particular patterns of fluctuation, but IgG- and IgA-ACPA to individual Cit-antigens often followed similar courses. Some new IgG- and IgA-ACPA, generally to different Cit-antigens however, arose at Relapse in 4 patients. The complexities of the ACPA response after rituximab may therefore reflect its ability to deplete or modify the function of parent B cell clones, which varies between patients. Although relapse following rituximab invariably follows naïve B cell exit from the bone marrow, these studies show that interactions between both 'new' and residual autoreactive memory B cells may be key to resumption of symptoms. The lack of identification of any immunodominant specificity suggests that the process of citrullination, rather than any particular Cit-antigen drives the autoimmune response in RA patients.



## 1. Introduction

The conversion of arginine residues to citrulline by peptidyl arginine de-iminases is a commonly observed post-translational modification of many proteins and is part of normal physiological

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processes. Citrullination of a variety of proteins is also commonly observed in the context of infiltrating cells in tissues from patients with inflammatory, infectious or autoimmune pathologies [1,2]. Smoking and periodontitis, now known to be environmental risk factors for the development of Rheumatoid arthritis (RA), both promote protein citrullination in mucosa. Autoantibodies directed against citrullinated moieties on a number of proteins or their derived peptide antigens (ACPA), are present in the majority of patients with RA and have a higher specificity than Rheumatoid factor (RhF) for the disease [3,4].

The ACPA response in RA patients comprises a mixture of antibodies recognizing over-lapping but also discrete citrullinated epitopes [5]. In the clinic, ACPAs are usually detected using combinations of cyclic citrullinated peptides (CCP) as a surrogate for binding to the variety of citrullinated proteins recognized by ACPA-containing sera. Citrullinated substrates for ACPA include matrix and serum proteins such as fibrinogen, vimentin, fibronectin, clusterin and innate ligands such as α-enolase, histones and heat shock proteins, but the full range of ACPA-reactivity is unknown.

Analysis of historic blood samples has revealed the presence of IgA- and IgG anti-CCP antibodies many months or years before the onset of clinical symptoms of RA [6,7]. IgA is the most common isotype of ACPA (as measured against synthetic CCP antigen) found in unaffected first-degree relatives of RA patients [8]. Autoantibodies targeting citrullinated proteins before positivity of the commercial anti-CCP test also have been reported [9]. Epitope spreading of the ACPA response together with raised levels of pro-inflammatory cytokines can precede development of clinical symptoms of synovitis and diagnosis of RA [10,11]. Although the measurement of individual fine specificities has not yet proved highly useful for diagnosis or predictive purposes, profiling combinations of specific citrullinated epitopes can potentially distinguish sub-groups of RA patients, and response to therapies as well as provide useful information regarding underlying pathogenic and etiological pathways [12–14].

B cell depletion therapy based on Rituximab, a monoclonal antibody to CD20, is now an established therapy for patients with RA. Meta-analysis has shown that patients seropositive for both RhF and ACPA achieved significantly greater benefit than other groups [15,16]. Clinical improvement following rituximab, however, is rather 'delayed' following removal of the majority of circulating B cells, often taking a few months for clinical benefit to be observed [17]. In RA synovia biopsied at 16 weeks post-rituximab, reduction in plasma cells, not B cells, correlated most closely with clinical response at 6 months. B cells, (when present), were reduced by 4 weeks post-treatment with a variable response at 16 weeks [18]. Relapse after rituximab has been shown to coincide with, or follow, resumption of exit of newly generated (naïve) B cells from the bone marrow to the periphery. This would suggest that it is the interruption of B cell maturation and differentiation, rather than removal of peripheral B cells per se that may be most important to its clinical success [19–21]. Rises in autoantibody production (RhF not anti-CCP) and differentiation or expansion of memory B cells (CD27+IgD+ un-switched and IgD-switched memory B cells) have been most closely linked with relapse [21–23]. Studies of the kinetics of serological and synovial responses therefore suggest that depletion of B cells with rituximab results in an indirect effect on autoantibody-committed daughter plasma cells, which could help explain the delay in response to rituximab treatment.

Despite the strong relationship between IgG antibodies to citrullinated antigens and disease expression, fluctuations of anti-CCP antibodies (using CCP2 ELISA) in RA patients have been of little practical value as a biomarker for disease activity, remaining at high levels in patients responding to therapy and rarely becoming seronegative, even after multiple cycles of B cell depletion therapy. This could be for many reasons but the proportion of 'pathogenic' ACPA within a heterogeneous population in serum is likely to be extremely small. With a view to possibly identifying immunodominant species associated with the kinetics of the clinical response during rituximab therapy, we used a Multiplex bead array system to follow the dynamics of the ACPA response to individual citrullinated antigens. The panel of antigens used was based on citrullinated proteins identified in RA synovia [24]. Results were analyzed at 3 key points in the treatment cycle namely Baseline, (before first cycle of rituximab), during the period of clinical improvement coinciding with adequate B cell depletion ('Depletion') in the peripheral blood and at disease flare (Relapse).

## 2. Patients and methods

### 2.1. Patients

16 consecutive patients with severe, active RA (DAS28 $\geq$ 5.1) fulfilling revised ACR/EULAR diagnostic criteria were included [25]. All patients who had undergone their first cycle of rituximab (2 weekly infusions of 1 g given 2 weeks apart), showed adequate depletion in the peripheral blood (<5 CD19+cells/μl) at 1–3 months, and had clinically responded (ΔDAS28 $\geq$ 1.2) within 5 months. Serum samples referred to as 'Depletion' were chosen from those available during the period of confirmed, adequate B cell depletion and when significant clinical improvement had also been established in the patient after rituximab. This was used rather than a fixed time point with respect to duration post-therapy due to the variation between patients in terms of timing of clinical improvement after achieving adequate B cell depletion.

Follow-up was either up until re-treatment, change in therapy or increased clinical symptoms associated with disease flare/relapse. All patients gave written informed consent to enter the study which was approved by the local ethics committee (H0715/18).

### 2.2. ACPA measurement using Multiplex bead array

A custom, bead-based, antigen array comprising CCP plus 29 RA-associated citrullinated, and 22 corresponding native/un-citrullinated antigens was used for measurement of antibodies in patient sera as described in detail elsewhere (assessed using mean fluorescence intensity-MFI) [10,26]. To compensate for number of binding sites per antigen, MFI were Z-normalized. Positive results were regarded as those with Z-score $\geq$ 1 above population mean, as previously described.

### 2.3. Statistical analysis

Spearman's Rank Correlation coefficient was used to describe relationships between IgG- and IgA-ACPA binding specificities. Z-normalized data were analyzed using Wilcoxon matched-pairs signed rank and Mann-Whitney rank sum tests as appropriate for paired and unpaired comparisons, and the paired *t*-test used to compare percentages. Descriptive statistics were used to describe patterns of antibody kinetics following rituximab.

## 3. Results

### 3.1. Clinical characteristics

Table 1 shows clinical demographics, concomitant medications and serum autoantibody status of the 16 RA patients studied. The median time to B cell return ($\geq$5 CD19+cells/μl) was 6.5 months (range 4–9 months) after the second rituximab infusion with time

**Table 1**
Patient demographics at baseline.

| Patient | Age (years)/sex | Disease duration (years) | CCP ELISA (class) | IgM-RhF (RAPA) | Months to B cell return | Months to flare/relapse | Pre-RTX therapy |
|---|---|---|---|---|---|---|---|
| 1 | 71/M | <1 | GM | 640 | 8 | 8 | HXQ + SFX + Pred/10 mg |
| 2 | 51/F | 5 | GMA | 1280 | 4 | 7 | MTX |
| *3* | [a]*56/M* | *19* | *GMA* | *>5120* | *6* | *7* | *None* |
| 4 | 62/F? | 4 | GMA | 1280 | 8 | 13 | MTX |
| 5 | 77/F | 25 | GMA | >5120 | 6 | 6 | MTX |
| *6* | *78/F* | *19* | *GM* | *320* | *7* | *Retreat* | *Etanercept* |
| *7* | *38/M* | *7* | *GMA* | *320* | *N/A* | *Retreat* | *MTX + adal* |
| *8* | *56/F* | *20* | *GM* | *640* | *6* | *Retreat* | *MTX + adal + Pred/20 mg* |
| 9 | 60/F | 20 | GMA | 2560 | 7 | 7 | MTX + SSZ |
| 10 | 74/F | 4 | GMA | 1280 | 7 | 7 | SSZ |
| 11 | 67/F | 22 | GM | 320 | 9 | 9 | MTX |
| *12* | *74/F* | *9* | *GMA* | *>5120* | *N/A* | *6* | *MTX* |
| 13 | 64/F | 21 | GMA | 320 | 7 | 10 | None |
| 14 | 64/F | 40 | GMA | neg | 6 | 6 | Adalimumab + Pred/7.5 mg |
| 15 | 61/M | 26 | GMA | >5120 | 6 | 7 | None |
| 16 | 77/F | 28 | M | 2560 | 6 | 7 | MTX |

[a] Samples from patients denoted in italic had only Pre-treatment and Depletion samples included. Abbreviations: G, IgG; M, IgM; A, IgA; RAPA, RA particle agglutination; adal, adalimumab; RTX, rituximab; HXQ, hydroxychloroquine; Pred, prednisolone; SSX sulphasalazine; MTX, methotrexate.

to flare of clinical symptoms ('Relapse') having a median of 7 months (range 6–13 months). For serial studies, samples from only 11/16 patients were available due to pre-emptive retreatment with rituximab before full disease flare or because of changes in their therapy due to non-RA associated symptoms.

### 3.2. MFI of baseline IgG- and IgA-ACPA specificities in Multiplex bead array

In Fig. 1, the full range of binding of class-switched ACPA to different citrullinated antigens can be appreciated in the 16 patients studied at baseline. MFI of binding of antibodies to CCP and to 29 other individual citrullinated moieties within sera from RA patients at Baseline are shown for IgG- and IgA-class ACPA in (A) and (B) respectively. Although some clustering of recognition of particular substrates by both IgG- and IgA-ACPA, each patient serum shows a different profile. Baseline MFI levels of IgG- and IgA-ACPA binding to individual epitopes were however significantly correlated (data not shown; Spearman's Rank: $R^2 = 0.75$; $p < 0.0001$) but with IgA-ACPA approximately 10 fold lower than those of IgG-ACPA. The greater intensity of binding to multiple epitopes by IgG-compared with IgA-ACPA was also evident in the heat maps shown in Figs. 2A and 3A respectively. In Figs. 2B and 3B, there was a clear lack of binding to corresponding un-citrullinated native antigens by IgG and IgA in the same RA sera. Comparisons between IgG- and IgA-ACPA should be treated with caution, however as the difference in MFI may be a function of the different isotype detection reagents used to separately detect IgG and IgA.

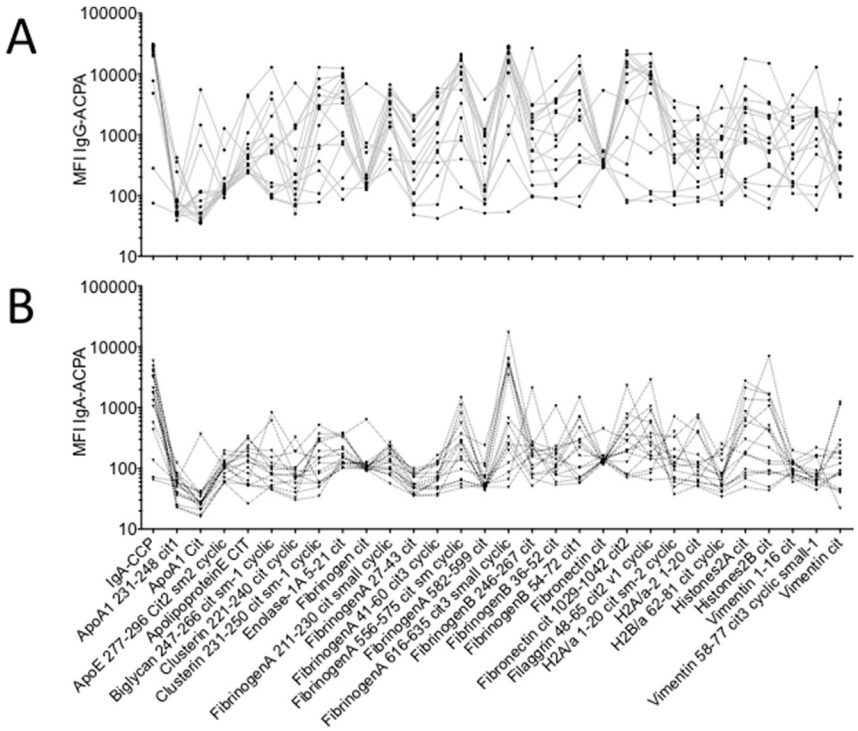


**Fig. 1. The mean fluorescence intensity (MFI) of binding of serum autoantibodies to CCP and 29 additional citrullinated antigens presented in the Multiplex Bead Array.** Lines join results found for IgG-class antibodies in (A) and for IgA in (B) by individual sera from each of the 16 patients with RA studied at baseline, before starting RTX therapy.

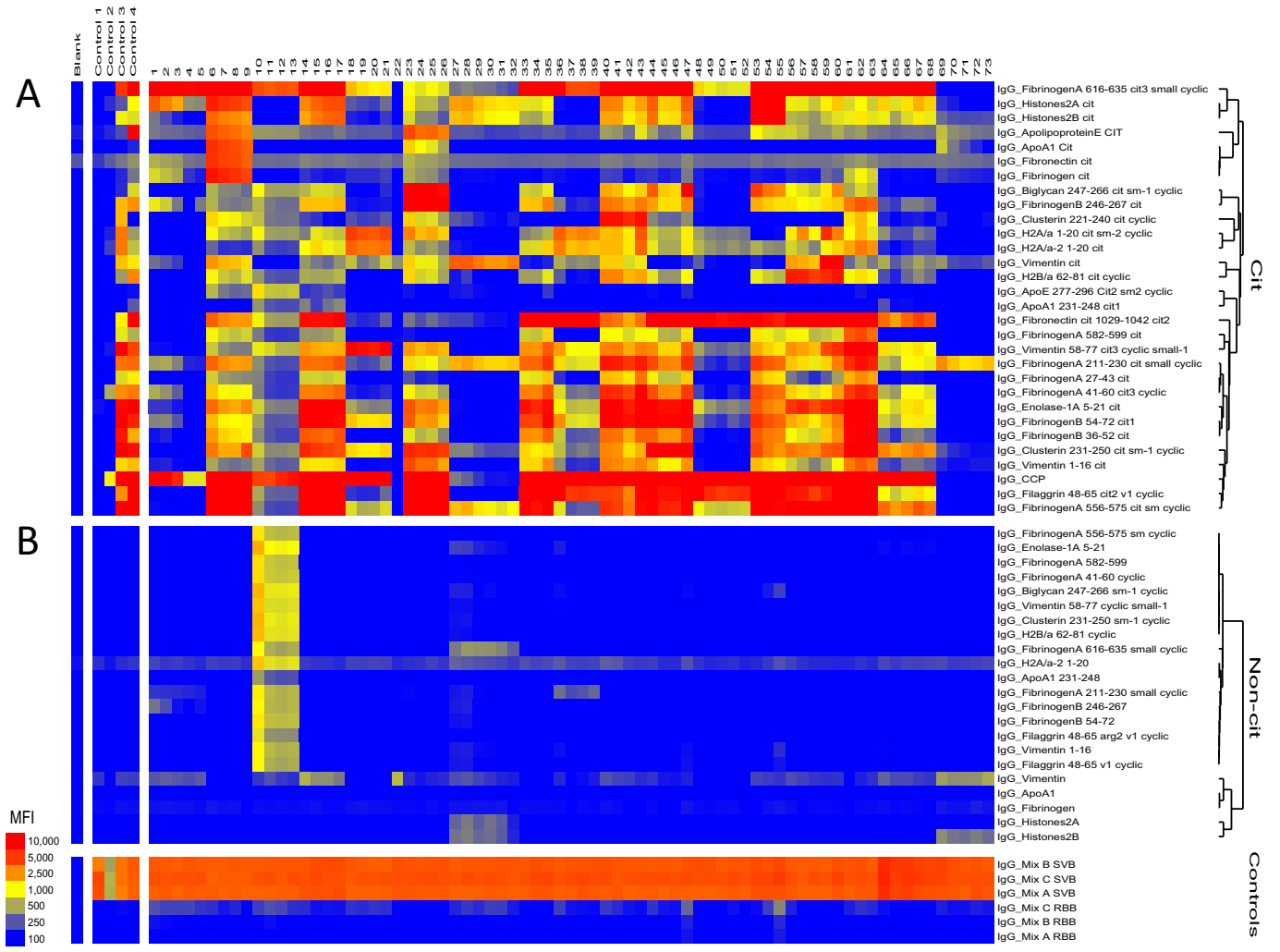


**Fig. 2.** A and B: Heat map showing MFIs of IgG present in serial samples from RA patients binding to multiple citrullinated (Cit) epitopes in Panel (A) and Non-citrullinated (Non-cit/native) epitopes in Panel (B). Individual specificities tested are shown on the right Y-axis. The sequence on top of the figure denotes firstly binding of a normal serum pool (Control 1) with Controls 2–4 consisting of pooled RA sera. The numbers that follow represent results of serial RA sera from patients during their initial cycle of Rituximab, from which data used in this manuscript was derived.

These reagents vary in affinity and degree of fluorophore conjugation and therefore differences in the MFI observed between the two isotypes may not allow direct comparison.

### 3.3. IgG- and IgA-ACPA at baseline and following achievement of B cell depletion with rituximab (Z-scores)

MFI data for each variable (antigen) was normalized and Z-scores calculated to allow different data to be compared. As Z-score measures the number of standard deviations an observation is away from the population mean (binding by panel of healthy control sera), Z-score of $\geq$1 was regarded as positive. Using this scoring system, the median number of citrullinated antigens recognized per patient was 3 (range 0–15) for IgG-ACPA and 4 (range 0–12) for IgA-ACPA (data not shown). The substrates most frequently recognized by IgG-ACPA were FibrinogenA 556–575 cit sm cyclic (by antibodies in sera from 6/16 patients) and FibrinogenA 616–635 cit3small cyclic (in 5 samples). For IgA-ACPA, sera from 5 patients contained antibodies with specificity for Clusterin 231–250 cit sm-1 cyclic with sera from 4 patients recognising Enolase-1A 5–21 cit (data not shown).

After rituximab and following reduction in peripheral B cell numbers in the peripheral blood (to <5CD19+B cells/μl) the median time to clinical improvement was 3 months (range 1–5). The mean % change from Baseline to Depletion in IgG-ACPAs was −21.4% whereas IgA-ACPAs tended towards a greater decrease from Baseline of −30.2% ($p = 0.09$, Unpaired *t*-test) (data not shown).

### 3.4. Kinetics of IgG and IgA responses to individual epitope specificities

Serial samples were available from 11/16 patients at all 3 key points (Baseline, Depletion, Relapse), due to confounding factors including change of therapy or infection. There were a total of 68 IgG- and 51 IgA-ACPA identified to individual Cit-substrates, as defined by having a positive Z-score at Baseline. Fluctuations in the mean Z-scores of IgG- and IgA-ACPA against the different Cit-substrates over the course of therapy are shown in Figs. 4 and 5. Results are presented divided into 2 plots for clarity. Fig. 4 therefore shows mean Z-scores for IgG-ACPA (A) and (B) IgA-ACPA which were directed against fibrinogen-derived substrates and in Fig. 5, mean Z-scores for IgG-ACPA (A) and (B) IgA-ACPA which were directed against antigens derived from substrates other than

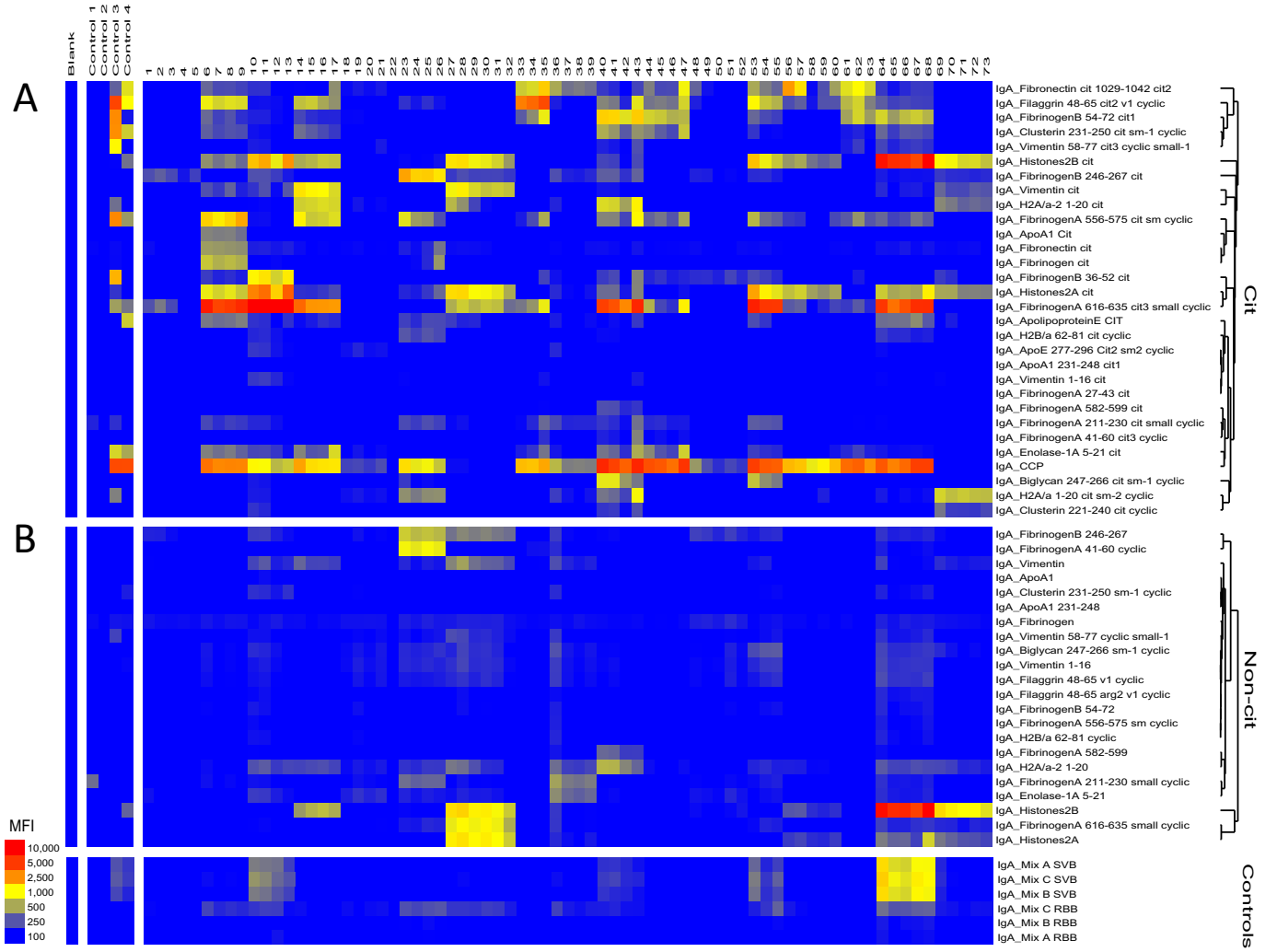


**Fig. 3.** A and B: Heat map showing MFIs of IgA present in serial samples from RA patients binding to multiple citrullinated (Cit) epitopes in Panel (A) and Non-citrullinated (Non-cit/native) epitopes in Panel (B). Individual specificities tested are shown on the right Y-axis. The sequence on top of the figure denotes firstly binding of a normal serum pool (Control 1) with Controls 2–4 consisting of pooled RA sera. The numbers that follow represent results of serial RA sera from patients during their initial cycle of Rituximab, from which data used in this manuscript was derived.

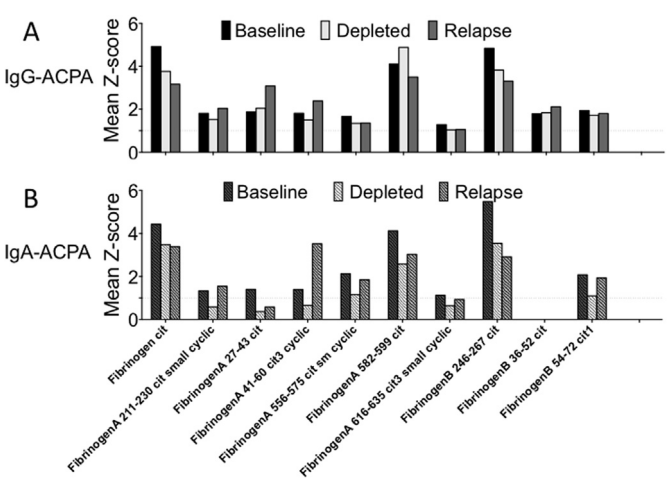


**Fig. 4. Mean Z-scores of ACPA responses to Cit-fibrinogen and Cit-fibrinogen-derived peptides in serial samples from 11 patients with RA at key points over the course of treatment with rituximab**. In A) IgG-ACPA and B) IgA-ACPA are shown, dotted line shows cut-off for positive response at baseline (Z-score ≥ 1).

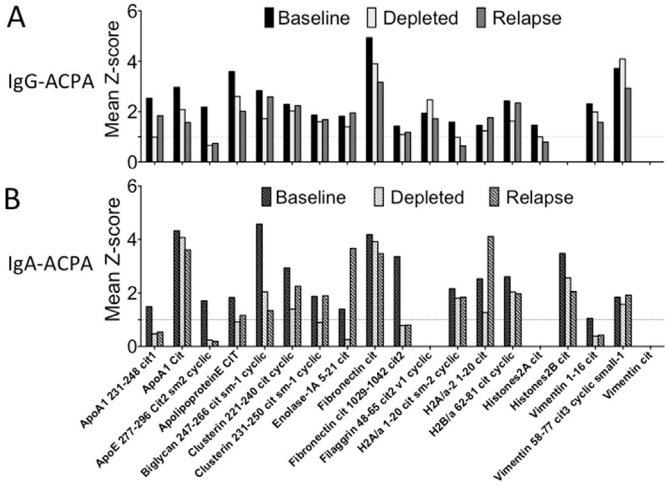


**Fig. 5. Mean Z-scores of ACPA responses to 20 Citrullinated antigens (excluding those derived from fibrinogens) in serial samples from 11 patients with RA at key points during treatment with rituximab**. In A) IgG-ACPA and B) IgA-ACPA are shown, dotted line shows cut-off for positive response at baseline (Z-score ≥ 1).

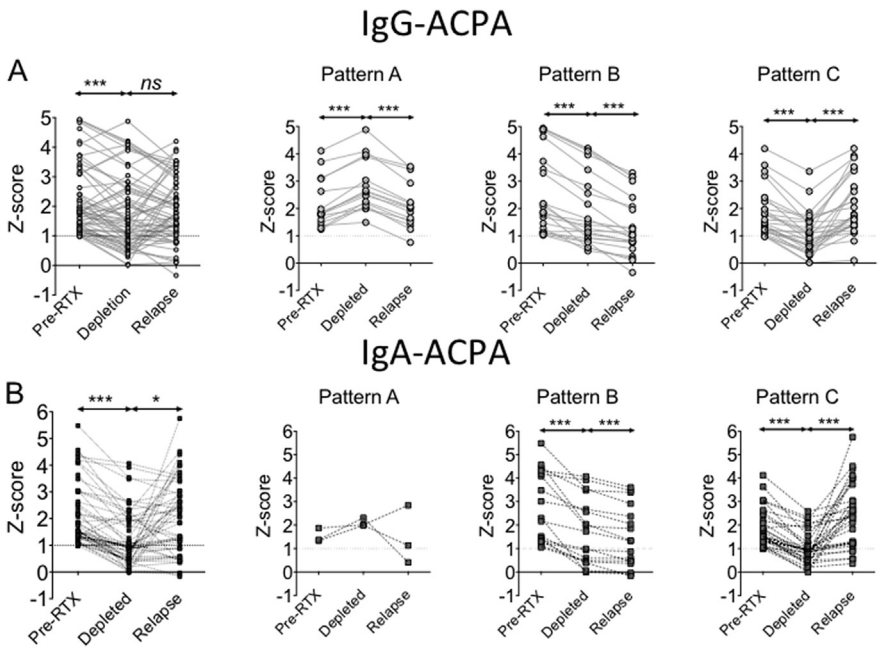


**Fig. 6. Z-scores of IgG-ACPA (A) and IgA-ACPA (B) present in serial samples from 11 RA patients at 3 key points during their initial cycle of rituximab**. Data shown in A and B were then selected on the basis of the pattern of response shown by individual autoantibody specificities. These were namely **Pattern A**: Z-score levels rose at Depletion with respect to Baseline; **Pattern B**, Z-scores fell after RTX and continued to fall to Relapse and **Pattern C**, where levels fell following RTX but then rose between Depletion and Relapse. Samples between key points were compared using Wilcoxon matched-pairs signed rank with $p < 0.0001^{***}$, $p < 0.001^{**}$ and $p < 0.01^{*}$.

fibrinogen. There was considerable diversity in the patterns of change of the ACPA response directed against different Cit-epitopes although IgG- and IgA-class ACPA tended to follow the same 'pattern' as one another to the same Cit-antigens.

### 3.5. Dynamics of IgG- and IgA-ACPA in serial samples after rituximab

In order to follow changes in Z-scores (scored as positive at Baseline) of ACPA to individual Cit-antigens, serial results were plotted against the key data points within a cycle of rituximab therapy. There was an overall decrease in median values for both IgG- and IgA-ACPA (Fig. 6A and B respectively) between Baseline and Depletion ($P < 0.0001$; Wilcoxon matched-Pairs Signed-Rank Test). Between Depletion and disease flare (Relapse), although IgA-ACPA showed a significant rise ($p = 0.01$; Wilcoxon matched-Pairs Signed-Rank Test), there was no significant change in median IgG-ACPA level. By following individual ACPA throughout the course of rituximab treatment, it was clear that ACPA could behave in several ways to rituximab based B cell depletion.

### 3.6. Three different patterns of ACPA response following rituximab

It was apparent from Fig. 6A and B that different ACPA seemed to 'respond' to rituximab treatment with different dynamics which could be divided into 3 basic patterns. Firstly, autoantibodies rising between Baseline and Depletion following rituximab (Pattern A) were separated from those which fell after rituximab. When a decrease in an individual ACPA was present between Baseline and Depletion, responses were the divided into those which continued to fall (Pattern B) or which rose between Depletion and Relapse (Pattern C). In Pattern A, after the initial rise in levels, antibodies to all specificities (with 1 exception within the IgA-ACPA), decreased to approximately baseline levels at Relapse. Following an initial fall from Baseline, a number of both IgG- and IgA-ACPA (29 and 31 respectively) were found to rise between Depletion and Relapse (Pattern C). The antigen specificities of ACPA showing each of the 3 patterns identified are shown in Supplementary Fig. 1. Within each of Patterns B and C, there was some concordance between IgG- and IgA-ACPA in terms of Cit-substrates recognized, but as responses to more that 20/30 antigens were recognized by each class of autoantibody, there was no apparent preference for any of the antibodies to particular Cit-specificities to either fall (Pattern B) or be associated with flare (Relapse).

### 3.7. Do ACPA to any new Cit-antigen specificities arise after rituximab?

Relapse following rituximab in RA patients is invariably associated with naïve B cells re-entering the peripheral circulation from the bone marrow [27]. Although sequencing data would be required to definitively answer whether predominantly new or old specificities expand at Relapse, we attempted to identify possible 'new' specificities by retrospectively identifying specificities found to be positive at Relapse which had been negative (Z-score < 1) at Baseline. Results are shown for IgG-ACPA in Fig. 7A and IgA-ACPA in Fig. 7B. Four Cit-substrates were recognized by both IgG- and IgA-ACPA. Although some new specificities did appear to be arising at relapse, most responses were modest, showing little real change as although Z-scores <1 were scored as 'negative', some binding could be detected in most examples. The possible implications of these results in this albeit small cohort of patients suggest that an overall impression that pre-existing ACPA specificities showed a more robust increase up to Relapse (Fig. 6, Pattern C) than the small number of possible 'new' specificities arising (Fig. 7). Thus the predominant source of ACPA post-rituximab seemed to be from the pre-existing memory B cell compartment.

## 4. Discussion

Using Multiplex bead array, we followed fluctuations in ACPA binding to citrullinated epitopes in clinically responding RA

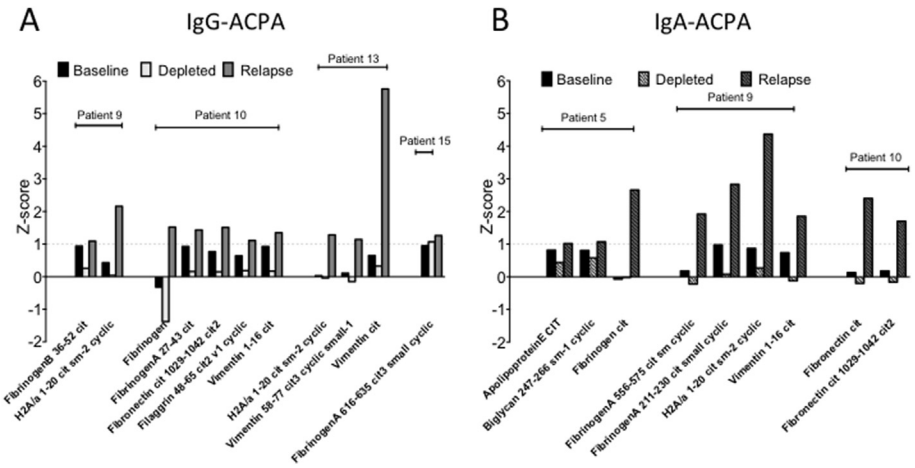


**Fig. 7. New antigen specificities arising co-incident with disease (RA) flare after rituximab**. The antigen specificities of autoantibodies giving a Z score of <1 at baseline which subsequently showed Z-score ≥ 1 associated with clinical flare are shown. Patients are numbered as in Table 1. In (A) IgG and (B) IgA class antibodies are shown.

patients during B cell depletion therapy based on rituximab. The aim was to follow changes in antibody responses to individual Cit-antigens during B cell depletion therapy in order to identify possible specificities associated with disease flare. Different patterns of autoantibody fluctuations following rituximab treatment were identified, highlighting the complexity of the ACPA response.

ACPA, measured using CCP or citrullinated fibrinogen as substrate in ELISA, significantly decrease following rituximab therapy. Using our bead-based assay, we confirmed overall falls in both IgG- and IgA-antibody levels to Cit-antigens following treatment. This has suggested that at least some ACPA are produced by short-lived plasmablasts continually being derived from $CD20^{+}$ B cell precursors [21,28]. Circulating plasmablasts capable of spontaneously producing ACPA are reported to be readily detectable in peripheral blood (PB) from RA patients [29]. Although overall, median levels of IgA- but not IgG-ACPA against multiple epitopes then showed a modest increase leading up to clinical relapse, responses to individual antigens revealed a more complex picture. Relative levels of some IgG- and IgA-ACPA which had decreased from baseline then continued to fall, implying that the parent clones of these activated ACPA-committed B cells were deleted or possibly, if resisting depletion, silenced. Depletion of activated (HLADRhi) B cells in PB and depletion of CD19+CD27+ memory B cells in PB and also in bone marrow in RA patients clinically responding to rituximab has been described in other studies [30].

Other clones of parent B cells were apparently spared as relative levels of some ACPA showed a more dynamic course and, after an initial fall, both isotypes of ACPA to a number of different citrullinated epitopes were found to rise between Depletion and Relapse; the antigen specificities of most of which were shared by both IgA- and IgG classes. Between patients however, there was no clear association between any particular Cit-epitope specificity and disease flare. These results suggested that several different paths could be taken by ACPA-specific B cell clones after encountering rituximab, possibly related to activation status at encounter, and the relative availability of pro-survival signals in the form of cytokines or protective niches [31].

Intriguingly, the levels of some (mostly IgG) ACPA rose rather than fell following rituximab before returning to levels similar to those at baseline. We cannot exclude the possibility that other fluctuations in levels had occurred between time points measured here. The transient increase of some ACPA following rituximab may be due to survival and differentiation of a proportion of undepleted memory B cell clones. This would support observations from *in vitro* studies of PB B cells incubated with sub-optimal levels of rituximab, which were found to be capable of proliferating for up to 2 cycles following T-cell dependent type stimulation. Rituximab-exposed B cells could also induce proliferation of T cells, especially of those with a Th2 phenotype [32]. Expansion or differentiation of un-depleted B cell clones co-stimulated by Th2 T cells could explain our findings. Rituximab may therefore affect the immune response not only through its activity as a potent B cell depleting agent, but also by subtly altering the functional properties of resistant B cells.

The proportion of B cells killed by rituximab, particularly in lymphoid organs and inflamed sites, will be affected by factors such as ability of the CD20 molecule to be modulated from the B cell surface which varies between patients and diseases [33], the presence of co-stimulatory molecules such as expression of CD80/86 and of pro-survival molecules such as VCAM [32] and cytokines in the micro-environment. Disease specific factors such as the relative proportions of autoimmune vs 'normal' B cells and their differentiation status may also contribute to the efficiency of depletion [31,34]. For example, naïve B cells ($CD27^{-}$), particularly in secondary lymphoid tissue, reportedly show greater sensitivity to depletion than class-switched memory B cells [31]. *In vitro* studies have also shown that pre-incubation with rituximab inhibited the proliferation of naïve B cells to a greater extent than CD27+ memory B cells [31]. As shown by rises in levels of some ACPA after an initial fall following B cell depletion, it would seem that memory B cells specific for ACPA can indeed persist and in some cases, differentiate to autoantibody production following naïve B cell exit from the bone marrow.

It is well known that the maintenance of antibody levels to infectious agents is a combination of antibody production by plasma cells and by proliferation and differentiation of memory B cell populations. This can be antigen independent, through activated T cells or microbial/innate stimuli, or be antigen-dependent, through re-exposure to inducing or cross-reactive antigen or through stimulation persisting antigen in the form of immune complexes. Unless out-competed by clones with a more pro-survival phenotype, memory B cells can therefore remain for many years. We found that in the autoreactive response to Cit-proteins, increased levels of some pre-existing autoantibody specificities were accompanied by relatively few novel ACPA specificities arising at disease flare. Despite apparently losing some clones of ACPA-committed B cells using rituximab our results suggest that ACPA clonotypes within each

individual patient remains relatively static, but capable of expansion coincident with flare. In patients with undifferentiated arthritis it has been reported that epitope spreading to multiple citrullinated antigens occurs during progression from undifferentiated arthritis to established RA, but after onset, epitope expansion appears to plateau [10], or be absent for up to 7 years follow-up [35]. The relative lack of change in the Cit-epitope repertoire recognized by ACPA after rituximab could therefore reflect asymmetric differentiation of memory B cell clones to both replenish the B cell memory pool and also undergo differentiation to autoantibody production. The survival of ACPA with certain specificities will therefore be determined as a result of competition between newly expanding B cell clones and resident memory B cells. In patients with a clinical response to rituximab lasting for long periods after B cell return, however, maturation of naïve B cells to memory phenotype and autoantibody production seems to be required before onset of Relapse [21,23].

The class-switch to IgG-ACPA is the strongest predictive factor for RA expression [36]. IgA-CCP are also often present in patients with recent onset RA, but are almost always associated with IgG-anti-CCP. ACPA of the IgA class are predominant in first-degree relatives (FDR) of RA patients [37]. There is also some evidence that IgG- and IgA-ACPA are associated with different risk factors [38]. Although serum IgA is largely bone marrow derived, mucosal immunity is strongly implicated in at least the initial stages of disease [39] with an association with the use of tobacco in both early RA patients and FDR [37]. The role of IgA-ACPA in disease onset or progression is as yet unclear. We found that at baseline, IgA- and IgG-ACPA against the 30 citrullinated targets were strongly correlated. Over the course of a rituximab cycle, there was also some similarity in patterns of response by both classes of ACPA. Interestingly, an α-enolase- derived peptide (enolase-1A 5–21 Cit) was recognized by both IgA and IgG-ACPA more frequently than other specificities, and was found to increase prior to relapse. ACPA to citrullinated enolase have been suggested to arise as a result of an immune response to a citrullinated product of microbial origin in the mucosa [40].

Effective recall responses to potential pathogens contain many 'layers' of the B cell memory process, with naïve and memory IgM B cells, class-switched IgG clones and plasma cells of different longevities playing a role in its maintenance [41]. Unravelling the situation with respect to disease establishment and resumption after rituximab is likely even more complex in spontaneous autoimmune diseases such as RA. Thus far, *in vitro* studies have shown that different autoreactive memory B cell populations from RA patients can either produce ACPA spontaneously or following T-dependent stimulation [20]. Naïve B cells exiting the bone marrow in (rituximab naïve) RA patients can reportedly recognise citrullinated epitopes, with some also derived from an inherently autoreactive immunoglobulin (VH) gene [42,43]. Whether autoimmunity in RA involves the persistent recruitment of T cells by naïve B cells emerging from the bone marrow, which may then provide help for memory B cells residing in protected sites in inflamed tissues, for example, or through some more subtle pathways remains to be explored.

The limitations of this study were the small number of patients included, all of whom had long-standing disease and treatment with a variety of DMARDS as well as failed/loss of response to at least 2 TNF inhibitors which may have affected the relative survival properties of their B cells. The range of ACPA substrates was not total. We aimed to minimize the influence of differences in the number of binding sites present on different citrullinated proteins by using Z transformation of the collected data.

## 5. Conclusion

The complexity of the ACPA response in patients with RA was revealed by following the behavior of individual ACPA specificities over a course of treatment with rituximab. Fluctuations in levels of IgG- and IgA-ACPA to 30 citrullinated antigens varied both between patients and over the course of therapy. These variations were not linked with particular Cit-antigen specificities. There was therefore no clear evidence for antigen-driven autoantibody production against any particular dominant protein epitope related to either matrix or intracellular derivation, moreover the response appeared to be against citrullination per se. Although our results do not provide any additional predictive information regarding biomarkers for relapse in RA patients treated with rituximab, it suggests possible new ways to investigate B cell biology and therapy.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2016.03.010.

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