The Anti-Citrullinated Protein Antibody immune response and its effector functions in Rheumatoid Arthritis

Kempers, A.C.

CHAPTER 3

The extensive glycosylation of the ACPA variable domain observed for ACPA-IgG is absent from ACPA-IgM and ACPA-IgG3

A. C. Kempers[1], L. Hafkenscheid[1], A. L. Dorjee[1], E. Moutousidou[1], F. S. van de Bovenkamp[2], T. Rispens[2], L. A. Trouw[1], M. van Oosterhout[3], T. W. J. Huizinga[1], R. E. M. Toes[1], H. U. Scherer[1].

[1] *Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands.*
[2] *Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, Amsterdam, The Netherlands.*
[3] *Department of Rheumatology, Groene Hart Ziekenhuis, Gouda, The Netherlands.*

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ABSTRACT

Anti-Citrullinated Protein Antibody (ACPA) IgG molecules from rheumatoid arthritis patients were recently demonstrated to contain highly sialylated *N*-linked glycans in the antigen-binding fragment (Fab). These *N*-glycans in ACPA-IgG Fab-domains conceivably result from a selective process and depend on the generation of *N*-glycosylation sites during somatic hypermutation. To date, it is unclear why such sites accumulate in ACPA-IgG, and at which time point and stage in B cell development ACPA acquire *N*-glycosylation sites. Here, we analyzed the presence of *N*-linked Fab glycans on ACPA-IgM, -IgG and -IgG subclasses to gain insight in the biological processes underlying the extensive Fab-glycosylation of ACPA-IgG. We performed detailed gel filtration chromatography of ACPA-positive RA sera and examined fractions of different molecular sizes for the presence of ACPA-IgG subclasses and their non citrulline-specific counterparts by ELISA. In addition, we isolated ACPA and non-citrulline Ig-containing fractions from synovial fluid or plasma samples of ACPA-positive patients to determine their molecular weight by western blot. ACPA-IgG consistently showed an increased molecular weight compared to non-citrulline specific IgG, consistent with the presence of Fab-glycans. In contrast to ACPA-IgG, no evidence was obtained for enhanced Fab glycosylation of ACPA-IgM and ACPA-IgG3. Further analysis indicated that ACPA-IgG1, 2 and to some extent 4, but not ACPA-IgG3, display increased molecular weights, pointing to the presence of *N*-linked glycans on ACPA-IgG1, 2 and 4. Together, these results indicate that the introduction of *N*-linked glycosylation sites in the variable domain of ACPA response does not occur at the IgM-stage, but rather takes place after the provision of T cell help also involved in isotype-switching.

INTRODUCTION

Recently, we described the presence of highly sialylated *N*-linked glycans in the antigen-binding fragment (Fab) of almost all anti-citrullinated protein antibody (ACPA) IgG molecules present in patients with rheumatoid arthritis (RA) (1, 2). These glycans could not be found on autoantibodies of several other specificities tested. Given the remarkably low avidity of polyclonal ACPA (3), this observation raises the intriguing possibility that citrullinated antigen-specific B cells could be selected based on the presence of glycans in the variable domain, rather than on affinity for their cognate antigen. *N*-glycosylation requires the presence of specific consensus sequences in the amino acid backbone of proteins (4). However, only few human germline Ig variable region genes encode for such sequences (5). So far, we could identify several *N*-glycosylation sites in ACPA-IgG Fab-domains using mass spectrometry, but none of these were encoded in the germline sequence (1). In addition, recent analysis of the ACPA B cell receptor repertoire suggests that the extensive presence of *N*-glycans in ACPA-IgG Fab-domains could indeed result from a selective process mediated by somatic hypermutations in germinal centers or germinal center-like structures (6). Moreover, the available data make it conceivable that the ACPA response matures under the influence of T-cell help, presumably in germinal centers, and that the introduction of *N*-glycosylation sites might be a crucial step by which B-cell tolerance checkpoints are breached. In this respect, it is relevant to note that ACPA are frequently present before the onset of clinical symptoms, and that recent epidemiological data indicate that the HLA-region is not a genetic risk factor for the development of ACPA, but rather associates with ACPA-positive RA (7, 8). Thus, the HLA-region, as proxy of the involvement of helper T-cells, contribute primarily to ACPA-positive RA rather than to ACPA-development in healthy subjects. From these observations, it is tempting to speculate that the role of T-cell help is found in the expansion of the ACPA-response taking place before disease development and that such T-cell help allows the introduction of *N*-linked glycosylation sites. If correct, additional glycosylation of the variable domain would be absent from ACPA-IgM as IgM expressing B-cells are considered to have received limited T-cell help if any, while other ACPA isotypes or IgG subclasses could carry glycans to a variable extent. Here, we tested this hypothesis to gain insight in the biological processes underlying the extensive Fab-glycosylation of ACPA-IgG.

METHODS

Patient samples

Synovial fluid (SF), plasma and serum samples were collected from ACPA-positive RA patients who visited the outpatient clinic of the rheumatology department at Leiden University Medical Center, Leiden, The Netherlands. All patients fulfilled the 1987 RA classification criteria and gave written informed consent. Additional SF samples were anonymously collected as rest material from joint paracentesis at the department of Rheumatology, Groene Hart Ziekenhuis, Gouda, The Netherlands.

Gel filtration chromatography

Gel filtration chromatography of sera of 6 ACPA-positive RA patients was performed by fast protein liquid chromatography (ÄKTA-FPLC), as described before (1). In short, 500 μl serum was filtered over a 0.2 μm filter and loaded on a HiLoad Superdex 200 column (GE Healthcare) at a flow of 1 ml/min. In total, 50-60 fractions of 1 ml were collected per serum sample. The (ACPA-) IgG subclass content of these fractions was determined by ELISA.

Isolation of ACPA

ACPA and non-citrulline reactive Ig fractions were isolated from 7 SF or plasma samples based on cyclic citrullinated peptide 2 (CCP2) affinity, as described before (1).

Lectin (SNA) affinity chromatography

Human serum samples were dialyzed against Tris-buffered saline (TBS, 10 mM Tris, 140 mM NaCl, pH 7.4) containing 0.1 mM $CaCl_2$ (TBS-Ca). A Tricorn column (GE Healthcare) containing 2 mL of *Sambucus nigra* agglutinin (SNA) agarose (Vector Laboratories) was equilibrated with 5-10 column volumes of TBS-Ca at 1 mL/min using an ÄKTAprime plus chromatography system (GE Healthcare). The dialyzed samples were diluted 8 times in TBS-Ca and applied to the column at 0.2 mL/min. After washing away unbound proteins with 5 column volumes of TBS-Ca at 0.2-0.5 mL/min, bound proteins containing sialic acid were eluted with 0.5 M lactose in 0.2 M acetic acid at 0.8 mL/min (7.5 column volumes). Between samples, the column was washed with 10 column volumes of TBS-Ca. The sialic acid containing fractions were immediately dialyzed against PBS overnight at 4 °C and all samples were stored at 4 °C before measuring IgM and IgG in ELISA.

Western blot

Eluted ACPA and non CCP2-specific Ig from flow through fractions were loaded under reducing conditions on a 10% mini-protean TGX gel and blotted by the Trans-Blot Turbo Transfer System (Bio-Rad). After 2 hours of blocking in 3% skim milk at room temperature

(RT), blots were incubated overnight at 4°C with polyclonal rabbit anti-human IgG-HRP (DAKO; 1:50.000), mouse anti-human IgG1-HRP (Life Technologies; clone HP6069; 1:2000), unlabelled rabbit anti-human IgM (DAKO; 1:10.000) or unlabelled mouse anti-human IgG3 (Nordic-Mubio; HP6080; 1:10.000). Afterwards, blots incubated with anti-human IgM and IgG3 were stained with a goat anti-rabbit or goat anti-mouse Ig-HRP (both DAKO; 1:5000) for 1 hour at RT. Antibody staining was detected by enhanced chemiluminescence (GE Healthcare) with the ChemiDoc Touch Gel Imaging System (Bio-Rad).

ELISA

IgG ELISA (Bethyl Laboratories) was performed according to the manufacturer's protocol. To detect ACPA-IgG, ELISA plates were coated with 1 μg/ml streptavidin and incubated with 1 μg/ml CCP2 biotin before adding samples. IgG was detected with rabbit anti-human IgG (DAKO 1:20.000). ELISAs detecting ACPA-IgG and non-ACPA-IgG subclasses were coated with 5 μg/ml streptavidin (Invitrogen) following incubation with 1 μg/ml biotinylated CCP2, or coated with 10 μg/ml $F(ab')_2$ goat anti-human IgG $F(ab')_2$ fragments (Jackson ImmunoResearch), respectively. Wells were incubated with gel filtration fractions, followed by detection with HRP labelled mouse-anti-human IgG1 or IgG4 (Life Technologies; 1:1000) or unlabelled mouse anti-human IgG2 (1:500) or IgG3 (1:1000; both Nordic-MUbio) and goat anti-mouse Ig-HRP (DAKO; 1:2000). To determine the amount of (ACPA-) IgG subclasses present in the gel filtration fractions, percentages were calculated from the OD_{415nm} values.

For the measurement of IgG subclasses in the SNA fractions, IgG and ACPA-IgG ELISAs were performed as described above and incubated with SNA- or SNA+ fractions. For the detection of non-ACPA IgG1 and IgG3 in the SNA fractions, ELISA plates were coated with $F(ab')_2$ goat anti-human IgG, $F(ab')_2$ fragment specific antibodies (Jackson ImmunoResearch 1 μg/ml) before incubation with the SNA fractions. Mouse anti-human IgG1-HRP (Life Technologies, 1:1000) and unlabeled IgG3 (Nordic MuBio, 1:1000) were used as secondary antibodies and goat anti-mouse (DAKO, 1:2000) as tertiary antibody for the IgG3 detection. For the ACPA-IgG1 and –IgG3 ELISAs, plates were coated with 5 μg/ml streptavidin and incubated with 1 μg/ml CCP2 before incubation with SNA- and SNA+ fractions. Mouse anti-human IgG1-HRP (Life Technologies 1:1000) and mouse anti-human IgG3 (Nordic MUbio 1:1000) followed by a goat anti-mouse Ig-HRP (DAKO 1:2000) was used to detect the amount of IgG1 and IgG3 present in the samples. Statistics for the SNA fractions were calculated with a paired Wilcoxon test.

RESULTS

ACPA-IgM is similar in size to its non-citrulline specific counterpart, in contrast to ACPA-IgG

Upon analysis by gel-filtration chromatography, ACPA-IgG, as compared to non-citrulline specific IgG from the same patient, was consistently detected in fractions containing higher molecular weight proteins, indicating an increased molecular weight in line with the presence of Fab-glycans (figure 1A, B (1)). As previously published, ACPA-IgG Fab-glycans are highly sialylated (2) and therefore likely to interact with SNA. Accordingly, ACPA-IgG could be strongly enriched upon SNA-purification (figure 1C), indicating the presence of sialic acid residues on ACPA-IgG. The type of column used for size exclusion chromatography did not allow us to detect a potential size shift for ACPA-IgM in the fractions, due to the large size of IgM molecules. However, ACPA-IgM molecules obtained by antigen-capture using CCP2 columns were similar in size as their non citrulline specific IgM counterparts as detected by western blotting, in contrast to ACPA-IgG which did display an shift in molecular weight (figure 1B).

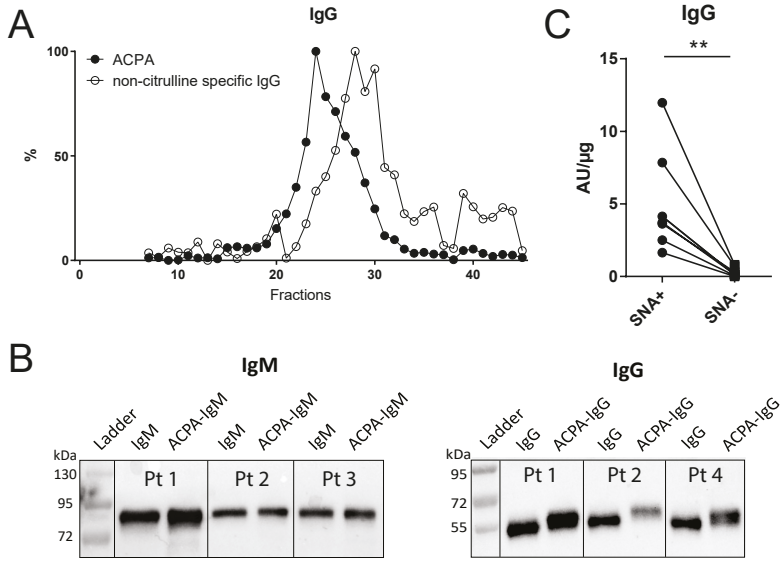

Figure 1. Molecular size analysis of ACPA-IgG and -IgM. (A) Presence of ACPA-IgG (black dots) and non-citrulline specific IgG (white dots) in gel-filtration chromatography fractions (lower fraction number indicates larger size; representative example of 6 donors). (B) Western blot of ACPA and non-citrulline specific control Ig isolated from patients (Pt) with established RA and stained for IgM and IgG (n=7). (C) Ratio of ACPA-IgG (in AU) per non-citrulline specific IgG (in μg) in SNA-positive (SNA+) and –negative (SNA-) fractions (obtained using sera from n=8 additional donors; non-parametric Wilcoxon test for matched-pairs, ** $p<0.01$).

A shift in molecular weight in ACPA-IgG subclasses

To determine whether Fab glycans are present in all subclasses of ACPA-IgG, we further examined the gel chromatography fractions for the presence of ACPA-IgG subclasses and their non citrulline-specific counterparts by ELISA. In all donor samples (n=6) analyzed, ACPA-IgG1 eluted earlier than total IgG1 (figure 2A). Indeed, western blot analysis confirmed the higher molecular weight of all ACPA-IgG1 samples (n=7; figure 2B) and the presence of ACPA-IgG1 in the SNA positive fraction upon SNA-purification (figure 2C). A similar shift in size was also present in fractions tested for the presence of ACPA-IgG2 (detected in 5 out of 5 ACPA-IgG2-positive donors (figure 2A)). In contrast, ACPA-IgG3 and non-citrulline specific IgG3 eluted in the same fractions and, additionally, showed a comparable molecular weight in western blot (figure 2A, B; 5 out of 5 ACPA-IgG3-positive donors). However, in contrast to what was expected based on size exclusion chromatography and western blot analysis, ACPA-IgG3 did not elute from the SNA-column in the flow-through fraction but was observed in the SNA-positive fraction. Finally, an increased size of ACPA-IgG4 could only be detected in 3 out of 6 donors. In these samples, ACPA-IgG4 eluted earlier than total IgG4, although the elution difference was not as apparent as observed for ACPA-IgG1 and ACPA-IgG2 (figure 2A). Together, these results suggest that ACPA-IgG1, 2 and to some extent 4, but not ACPA-IgG3, have increased molecular weights, indicating the presence of *N*-linked glycans on ACPA-IgG1, 2 and 4.

DISCUSSION

Extensive Fab glycosylation is a recently discovered unique molecular feature of ACPA-IgG, which may affect immunological functions such as antigen binding, fine-specificity and interaction with lectins (5). Recent analysis of the B cell receptor repertoire of ACPA-expressing B cells demonstrated that the *N*-glycosylation sites required for linkage of glycans to the protein backbone are introduced into the variable domain of ACPA-IgG upon somatic hypermutation (6). To date, it is unclear why such sites accumulate in ACPA-IgG, and at which time point and stage in B cell development ACPA acquire these *N*-glycosylation sites. Here, we analyzed the presence of *N*-linked Fab glycans on ACPA-IgM, -IgG and -IgG subclasses. Based on our analysis of *N*-glycosylation sites derived from ACPA-IgG (1, 6), we hypothesized that such sites would be absent from ACPA-IgM, while we expected to find Fab glycosylation of all ACPA-IgG subclasses. Interestingly, our data presented here indicate that ACPA-IgM and ACPA-IgG3 do not carry additional Fab glycans, as no increase in molecular weight could be detected when compared to non citrulline-specific IgM and IgG3. In contrast, an increased molecular mass of ACPA-IgG1

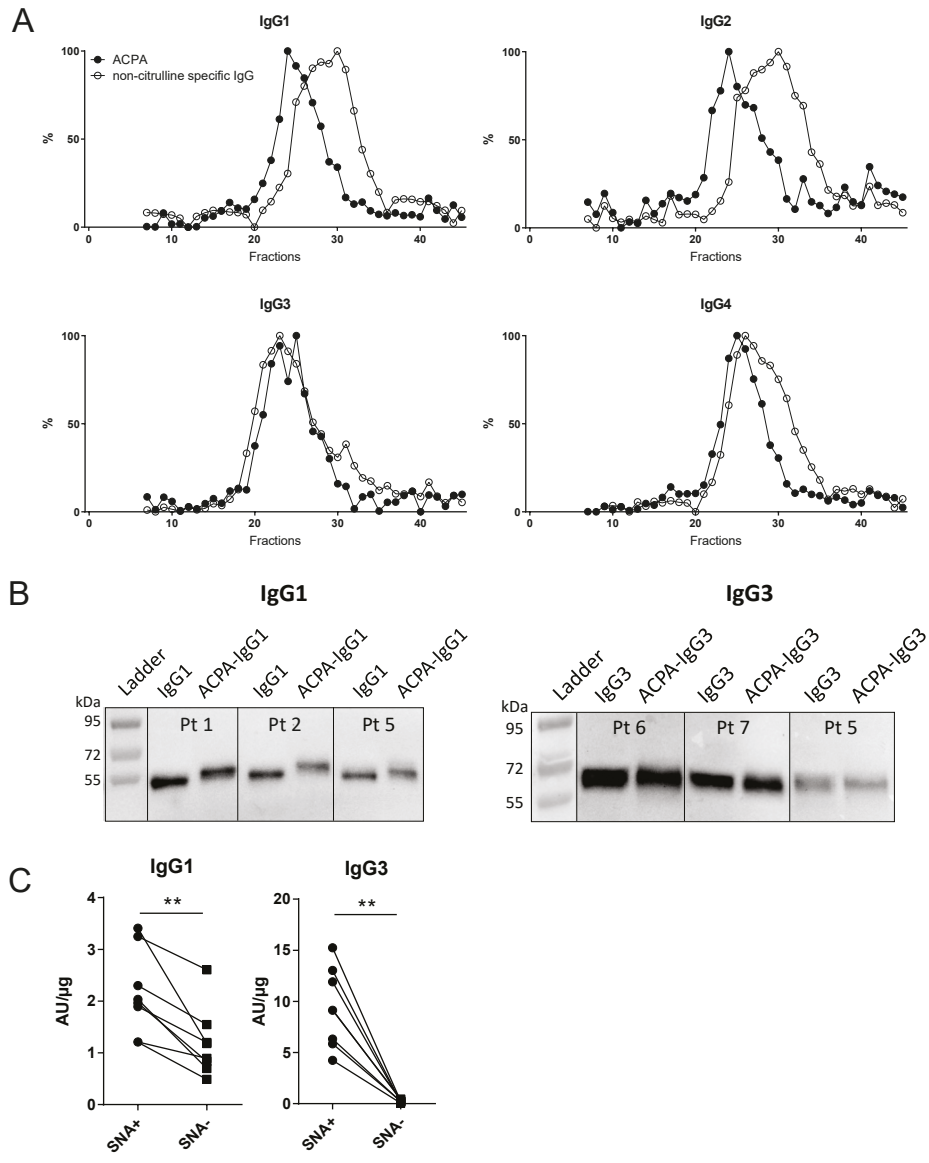

Figure 2. Higher molecular weight is detected for ACPA-IgG1, 2 and 4, but is absent with ACPA-IgG3. (A) ACPA-positive RA sera (n=6) were fractionated by gel filtration chromatography and the presence of (ACPA-) IgG subclasses were measured by ELISA. ACPA (black dots) and non-citrulline specific IgG (white dots) of each IgG subclass are shown (representative data of one donor). (B) ACPA and non-citrulline specific fractions were isolated from synovial fluid or plasma (n=7) of ACPA-positive RA patients by affinity chromatography. ACPA and FT samples were loaded under reduced conditions on western blot and detected with anti-human IgG1 or IgG3 antibodies. Results of 3 patients are shown. (C) Ratio of ACPA-IgG1 and –IgG3 (in AU) per non-citrulline specific IgG1 and IgG3 (in μg) in SNA-positive (SNA+) and –negative (SNA-) fractions (obtained using sera from n=8 additional donors; non-parametric Wilcoxon test for matched-pairs, ** $p<0.01$).

(6 out of 6 donors) and of ACPA-IgG2 (5 out of 5 donors) was observed, in comparison to their respective, non-citrulline specific counterparts. A variable, less pronounced increase in mass was observed for ACPA-IgG4 (3 out of 6 donors). Together, these data suggest that ACPA-IgG1, 2 and to some extent 4, harbour Fab-linked *N*-glycans, while ACPA-IgM and ACPA–IgG3 do not appear to carry additional Fab glycans in the variable domain.

The absence of additional Fab glycans from ACPA-IgM is in line with the hypothesis that *N*-glycosylation sites in the variable domain of ACPA-Ig are generated by somatic hypermutation in germinal centers (6). Indeed, synovial fluid and peripheral blood-derived monoclonal ACPA showed a high degree of somatic hypermutation, supporting this notion (6, 9). The acquisition of *N*-glycosylation sites by almost all ACPA-IgG molecules is striking. It suggests that ACPA mature under the influence of T cell help required to induce activity of the enzyme activation-induced cytidine deaminase (AID). Given the concurrent lack of affinity/avidity maturation observed for ACPA-responses from pre-disease to disease, these observations indicate that citrullinated antigen-specific B cells acquire a selective advantage due to hyperglycosylation of their respective B cell receptor. Therefore, additional in-depth analysis of the variable region repertoire usage of these B cells and of the exact localization of *N*-glycosylation sites in ACPA variable regions is needed to gain further insight into the breach of tolerance and/or breakdown of peripheral “auto-immune checkpoints”.

While ACPA-IgG1 and -IgG2 showed a clear “size-shift” compatible with extensive Fab glycosylation, this was not observed for ACPA-IgG3, both in size exclusion chromatography and western blot. A likely explanation for the absence of Fab glycans on ACPA-IgG3 could be the location of the IgG3 heavy chain in the front position within the IGH gene locus. Due to this localization, IgG3 is the first IgG subclass to be produced upon class-switch recombination of the B cell receptor, and is associated with less frequent mutations in the VDJ genes compared to the other subclasses in the IGH locus (10, 11). Given that both class-switch recombination and somatic hypermutation are mediated by AID, it is possible that ACPA-IgG3 is expressed prior to the accumulation of *N*-glycosylation sites in the variable regions of other IgG subclasses. However, whereas the shift in molecular size for ACPA-IgG1 corresponded to its presence in the SNA-positive fraction, we unexpectedly observed also ACPA-IgG3 in the SNA-positive fraction. These data suggest that ACPA-IgG3 may carry sialylated glycans at other positions in the antibody molecule, independent of variable domain glycosylation. One possibility that may explain this finding is the presence of *O*-glycosylation in the IgG3 hinge region as well as increased sialylation of the Fc-sugars (12). Further research is necessary to

confirm the absence of glycans in the ACPA-IgG3 variable domain and to explain the (presumably) enhanced sialylation of ACPA-IgG3 in other regions.

The “size-shift” detected for IgG4 was more subtle than that for the other subclasses. IgG4 antibodies are dynamic molecules which can exchange antibody half-molecules in a process called Fab-arm exchange. In this way, bi-specific antibodies are generated which express two different antigen recognition sites (13). As multiple IgG4 antibodies against other antigens are generally present in most individuals, it can be hypothesized that bi-specific ACPA-IgG4 antibodies might occur with Fab glycans on only one half-molecule. This could provide an explanation for the subtle shift in size that is observed for ACPA-IgG4 (figure 2A).

Among the different isotypes, ACPA-IgG(1) and ACPA-IgA are the most prevalent in ACPA positive RA (14, 15). Unfortunately, we were not able to reliable verify the presence of Fab glycans for ACPA-IgA due to the conformation of IgA, which consists of monomers, dimers and molecules with and without a J-chain. This conformation leads to differences in molecular weight and several “neighboring” peaks in size exclusion chromatography, which make the interpretation of shifts in size more difficult to interpret. We expect that the analysis of the B cell receptor repertoire of ACPA-IgA expressing B cells will clarify this question.

The interpretation of our results is based on the notion that the presence/absence of Fab glycans can be used as a proxy for the presence/absence of *N*-glycosylation sites, which limits our analysis. Although our data so far support this view, it is possible that *N*-glycosylation sites are present that are not used for the addition of glycans due to, for example, steric hindrance. However, sequencing data of the BCR repertoire of citrullinated antigen-specific B cells available so far demonstrated that over 90% of the ACPA-IgG B cell receptors contained one or more *N*-glycosylation sites, clearly indicating that most of the *N*-glycosylation site are occupied (6).

To conclude, we here show that enhanced Fab glycosylation is not detected on ACPA-IgM and ACPA-IgG3 while it is present on ACPA-IgG1 ,2 and 4. The absence of a molecular “size-shift” for ACPA-IgM and ACPA-IgG3 suggests that these ACPA isotypes lack additional glycosylation and glycosylation sites in the variable region. These results are compatible with the notion that the acquisition of Fab-glycans in the variable domain of ACPA-IgG reflects a T-cell dependent process in the development of citrullinated antigen-specific B cells in RA. In future studies, it will be crucial to understand the potential selective survival signals these glycans confer to ACPA-expressing B cells.

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