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Rombouts, Y.; Willemze, A.; Beers, J.J.B.C. van; Shi, J.; Kerkman, P.F.; Toorn, L. van; ... ; Toes, R.E.M.

EXTENDED REPORT

Extensive glycosylation of ACPA-IgG variable domains modulates binding to citrullinated antigens in rheumatoid arthritis

Yoann Rombouts,[1,2] Annemiek Willemze,[1] Joyce J B C van Beers,[3] Jing Shi,[1] Priscilla F Kerkman,[1] Linda van Toorn,[1] George M C Janssen,[4,5] Arnaud Zaldumbide,[6] Rob C Hoeben,[6] Ger J M Pruijn,[3] André M Deelder,[2] Gertjan Wolbink,[7,8] Theo Rispens,[7] Peter A van Veelen,[4] Tom W J Huizinga,[1] Manfred Wuhrer,[2] Leendert A Trouw,[1] Hans U Scherer,[1] René E M Toes[1]

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For numbered affiliations see end of article.

Correspondence to
Dr Yoann Rombouts, Department of Rheumatology, C-05-61, Leiden University Medical Center, Postbus 9600, Leiden 2300 RC, The Netherlands; y.j.p.c.rombouts@lumc.nl

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ABSTRACT

Objectives To understand the molecular features distinguishing anti-citrullinated protein antibodies (ACPA) from 'conventional' antibodies in rheumatoid arthritis (RA).

Methods Serum of ACPA-positive RA patients was fractionated by size exclusion chromatography and analysed for the presence of ACPA-IgG by ELISA. ACPA-IgG and non-citrulline-specific IgG were affinity purified from serum, plasma and/or synovial fluid and analysed by gel electrophoresis. Electrophoresis bands were excised, enzymatically digested and analysed by mass spectrometry. Binding affinity to citrullinated antigens was measured by ELISA and imaging surface plasmon resonance using recombinant monoclonal ACPA with molecular modifications.

Results In all donor samples studied (n=24), ACPA-IgG exhibited a 10–20 kDa higher molecular weight compared with non-autoreactive IgG. This feature also distinguished ACPA-IgG from antibodies against recall antigens or other disease-specific autoantibodies. Structural analysis revealed that a high frequency of N-glycans in the (hyper)variable domains of ACPA is responsible for this observation. In line with their localisation, these N-glycans were found to modulate binding avidity of ACPA to citrullinated antigens.

Conclusions The vast majority of ACPA-IgG harbour N-glycans in their variable domains. As N-linked glycosylation requires glycosylation consensus sites in the protein sequence and as these are lacking in the 'germline-counterparts' of identified variable domains, our data indicate that the N-glycosylation sites in ACPA variable domains have been introduced by somatic hypermutation. This finding also suggests that ACPA-hyperglycosylation confers a selective advantage to ACPA-producing B cells. This unique and completely novel feature of the citrulline-specific immune response in RA elucidates our understanding of the underlying B cell response.

INTRODUCTION

Rheumatoid arthritis (RA) is a severely destructive inflammatory disorder affecting ~1% of the population.[1] The majority of RA patients (60–70%) harbour anti-citrullinated protein autoantibodies (ACPA). ACPA represent highly disease-specific biomarkers of important diagnostic and prognostic value, with ACPA-positive patients being at higher risk for rapidly progressive, destructive and systemic disease.[2 3] ACPA strongly associate with polymorphisms in the human leukocyte antigen (HLA) region, which indicates a role for antigen-specific T cells in the formation and/or the evolution of this immune response.[4] Initially, the ACPA response generates polyclonal antibodies at low level and can be present for many years in the absence of clinical symptoms.[5] Upon a putative trigger, the ACPA epitope recognition repertoire broadens, more isotypes are being used and ACPA serum levels rise.[6 7] Although this expansion of the immune response occurs well before the onset of clinically detectable arthritis, subclinical synovitis and bone loss may already be present at the pre-disease stage.[8] Thus, the event that initiates a broadening of the citrulline-specific immune response could mark a crucial moment upon which the inflammatory process becomes self-perpetuating and, potentially, irreversible. Therefore, it is of great relevance to understand the events leading to the generation/perpetuation of the ACPA response at the molecular level.

A growing body of evidence has suggested mechanisms by which ACPA could be involved in driving the pathogenic processes found in RA. These include activation of immune effector cells,[9–12] triggering of complement pathways[13] and direct effects on bone metabolism.[14] Most of these effects relate to in vitro assays, however, and many aspects of the in vivo ACPA response remain yet to be defined. For instance, it has been a puzzling observation that the citrulline-specific immune response, despite signs of extensive isotype switching and somatic hypermutation, generates a pool of antibodies of remarkably low avidity.[15] As this and other aspects are poorly understood, we set out to characterise ACPA molecules in detail to better understand ACPA-mediated biological effects, to gain insight into ACPA structure–function relationships and to perhaps be able to conclude on the underlying B cell response.

We found that in all patient samples analysed (n=24), the vast majority of ACPA-IgG molecules exhibit a higher molecular weight in comparison with other autoantibodies or non-autoreactive IgG.

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Structural analyses demonstrated that the increase in weight is due to the presence of N-glycans in the ACPA variable domains. Finally, these variable domain N-glycans were found to modulate the binding of ACPA to citrullinated antigens.

METHODS

Additional information on material and methods is available as online supplementary file.

Patient samples

Serum, plasma and synovial fluid samples from ACPA-positive RA patients were collected at the outpatient clinic of the rheumatology department at Leiden University Medical Center (LUMC). All RA patients fulfilled the American College of Rheumatology 1987 revised criteria for the classification of RA and gave written informed consent.[16] Treatment included disease-modifying antirheumatic drugs, biological agents and glucocorticoids. For detailed RA patient characteristics, see online supplementary table S1. Sera from patients suffering from systemic lupus erythematosus (n=4), Sjögren's syndrome (n=3), coeliac disease (n=3) or myasthenia gravis (n=3) served as controls. Permission for conduct of the study was obtained from the LUMC ethical review board.

Gel filtration chromatography and ELISA

Gel filtration was performed by fast protein liquid chromatography (ÄKTA-FPLC equipped with a Hiload Superdex 200 (GE Healthcare), see online supplementary file). Chromatography fractions were analysed by ELISA to detect total IgG (Bethyl Laboratories), anti-CCP2-IgG (Immunoscan RA Mark 2; Eurodiagnostica), anti-dsDNA-IgG (Anti-dsDNA DIASTAT, Eurodiagnostica) and anti-SSA-IgG (SS-A p200 WIESLAB, Eurodiagnostica), according to the manufacturer's instructions. Anti-citrullinated fibrinogen (Fib), anti-citrullinated myelin basic protein (MBP), anti-tetanus toxoid, anti-muscle-specific tyrosine kinase (MuSK) and anti-transglutaminase (TGA) ELISA were performed using in-house protocols, as described previously.[15 17–20]

Structural analysis

(ACPA)-IgG (isotypes 1, 2 and 4) were isolated from RA patient samples by affinity purification using FPLC (ÄKTA, GE Healthcare) as described (see online supplementary file and refs. [21 22]). Antibody F(ab′)$_2$ and Fc fragments were generated by antibody digestion with ideS (FabRICATOR; Genovis) and purification of the resulting fragments on CaptureSelect affinity beads (Thermo Fisher). Following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), heavy and light chains (HC/LC) of (ACPA)-IgG were digested in-gel by PNGase F to release N-glycans. Glycans were labelled with 2-aminobenzoic acid (2-AA), purified by hydrophilic interaction chromatography solid-phase extraction (HILIC-SPE) and characterised by matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS).[23 24] N-glycosylation sites within ACPA were identified by in-gel PNGase F-assisted ^{18}O-labelling of N-glycosylation sites,[25] followed by in-gel trypsin digestion and nanoHPLC MS/MS analysis of the digested peptides (see online supplementary file).[26] 18O-peptide sequences identified were confirmed by matching tandem mass spectra of gel-eluted peptides with those of their synthetic counterparts.

Antigen-binding assay

Monoclonal ACPA (mACPA) were produced in human embryonic kidney (HEK) 293T cells (see online supplementary file). Binding of mACPA to CCP2 was analysed by ELISA using undiluted and diluted (1/10 to 1/250) HEK cell culture supernatants. Background signal corresponding to binding of mACPA to the arginine variant of CCP2 was subtracted when appropriate. Imaging surface plasmon resonance (iSPR) was performed as described in the online supplementary methods.[27]

RESULTS

ACPA-IgG exhibit an increased molecular weight

To study characteristics of the ACPA molecules, we fractionated serum of ACPA-positive RA patients (n=7) using size exclusion chromatography. Unexpectedly, ACPA-IgG eluted from the chromatography column in fractions preceding those containing most other IgG molecules, indicating an increased molecular weight (figure 1A). This was observed when CCP-2 (figure 1A), citrullinated fibrinogen (cit-Fib) or citrullinated MBP (cit MBP) antigens (figure 1B) were used to detect the presence of ACPA. In contrast, IgG against recall antigens from the same individuals, such as tetanus (figure 1A), *Escherichia coli* and diphtheria toxin, co-eluted in fractions corresponding to 'conventional' IgG. Moreover, no elution shift was observed for a number of other autoantibodies (anti-double-stranded DNA (anti-dsDNA), anti-SSA, anti-TGA and anti-MuSK) derived from serum of patients with systemic lupus erythematosus (n=4), Sjögren's syndrome (n=3), coeliac disease (n=3) or myasthenia gravis (n=3), respectively (figure 1C). The particular elution pattern of ACPA was observed for all sera tested, regardless of rheumatoid factor status (figure 1D). This intriguing observation indicated that ACPA-IgG are either larger than 'conventional' IgG or are bound by a factor causing the elution shift in size exclusion chromatography. To confirm our findings and to differentiate between these two possibilities, we affinity-purified ACPA-IgG (IgG1, 2 and 4 subclasses) and non-citrulline-specific IgG (depleted of ACPA) from serum (n=7), plasma (n=7) or synovial fluid (n=3) of RA patients followed by SDS-PAGE. Under non-reducing conditions, we again observed a higher molecular weight (corresponding to an additional 10–20 kDa) for purified ACPA-IgG compared with ACPA-depleted IgG from the same donors (figure 2A).

The higher molecular weight of ACPA-IgG is due to extensive Fab-linked N-glycosylation

To locate and identify possible post-translational modifications that could be responsible for the increased molecular weight of ACPA, we analysed ACPA-IgG F(ab′)$_2$ fragments and Fc fragments by SDS-PAGE. While no mass difference was observed for the Fc fragments, the F(ab′)$_2$ portion of ACPA-IgG showed a higher molecular weight than 'conventional' IgG (figure 2A). Under reducing conditions, this observation was maintained, with both HC and LC displaying several bands of increased molecular weight, thereby excluding non-covalent linkage of one or more additional proteins to the ACPA molecule (figure 2B). Of note, the presence of one or more HC and LC bands was found in ACPA-IgG of all donor samples analysed (n=17, figure 2B and see online supplementary figure S1) and indicated that the increase in molecular weight could be due to the variable addition of one or more defined structures to the ACPA-IgG molecule. As such shifts in the electrophoretic profile of antibodies have previously been attributed to the presence of Fab-linked N-glycans,[28–31] we hypothesised that glycans could be responsible for the mass shift of ACPA-IgG. To test this, purified (ACPA)-IgG molecules were treated with PNGase F and compared with untreated antibodies by SDS-PAGE under reducing conditions (figure 3A). As expected, PNGase F digestion of non-ACPA IgG decreased the apparent molecular weight of the HC due to

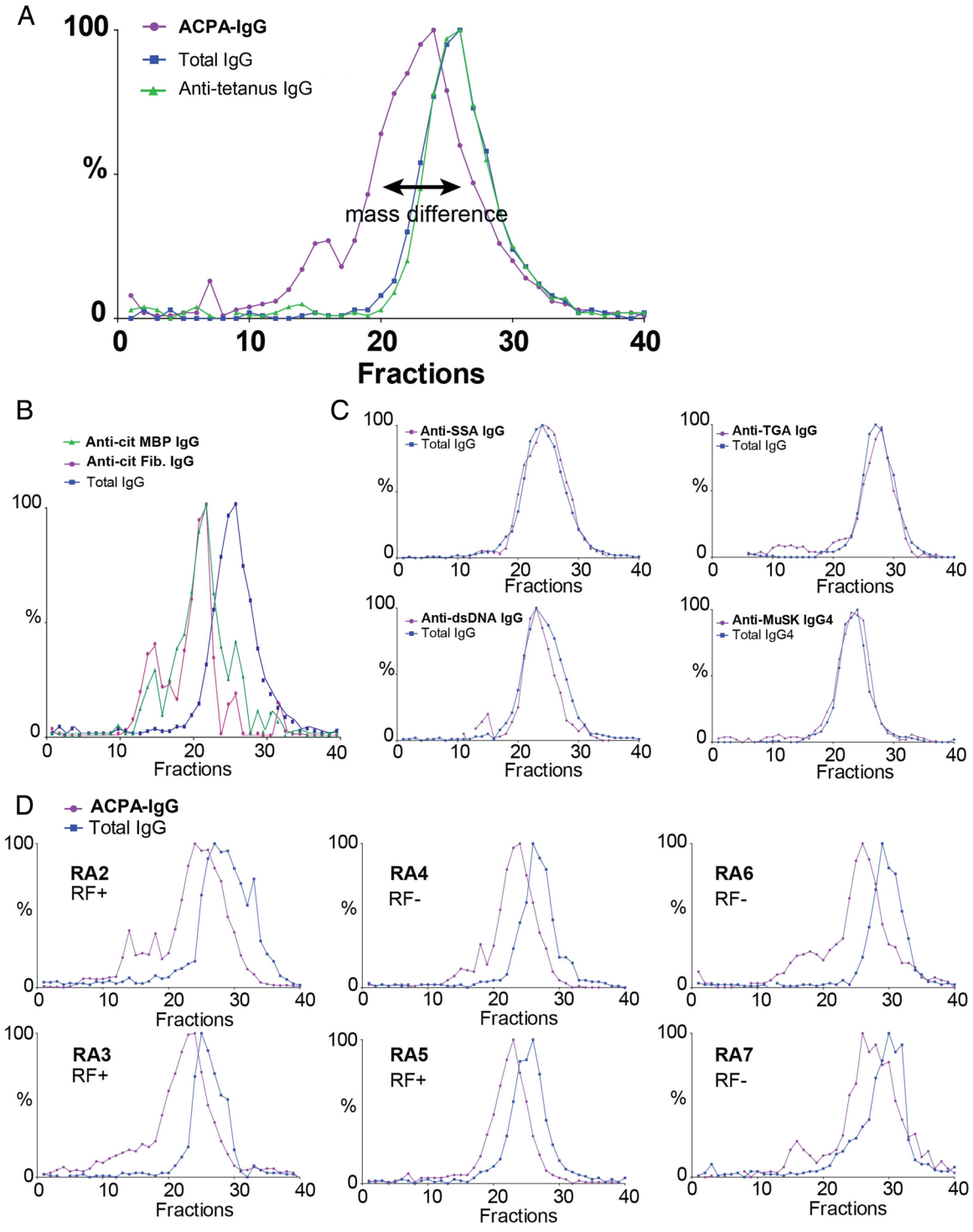

Figure 1 Anti-citrullinated protein antibodies (ACPA)-IgG exhibit a higher molecular weight than other IgG molecules. (A) Rheumatoid arthritis (RA) patient (RA1) serum fractionation by gel filtration chromatography, followed by ELISA detection, shows that ACPA-IgG (anti-CCP2 IgG antibodies) elute earlier than other IgG molecules, including antibodies against recall antigens (ie, anti-tetanus). (B) The earlier elution was also observed for other ACPA-IgG antibodies directed against citrullinated fibrinogen (cit-Fib) and citrullinated myelin basic protein (MBP). (C) Other IgG autoantibodies (Anti-SSA, Anti-dsDNA, Anti-TGA and Anti-MuSK) do not show this shift and co-elute with total IgG. Anti-SSA, anti-SSA p200 peptide; Anti-dsDNA, anti-double-stranded DNA; Anti-TGA, anti-transglutaminase; Anti-MuSK, anti-muscle-specific tyrosine kinase. (D) The higher molecular weight of ACPA-IgG is independent of rheumatoid factor (RF) serology. Both ACPA-IgG from serum of RF^+ and RF^- RA patients have a higher molecular weight than other IgG molecules.

release of the constitutive Fc-glycans, while no effect was observed on the electrophoretic mobility of the LC. In contrast, PNGase F treatment completely abolished the presence of additional electrophoretic HC and LC bands of ACPA-IgG, indicating that the higher molecular weight observed for ACPA-IgG is indeed due to additional N-glycans in the Fab-portion. To confirm and substantiate this finding, electrophoretic bands of ACPA-IgG and ACPA-depleted IgG were excised and N-glycans were released by in-gel PNGase F digest followed by MALDI-TOF-MS (figure 3B and see online supplementary figure S2). As expected, analysis

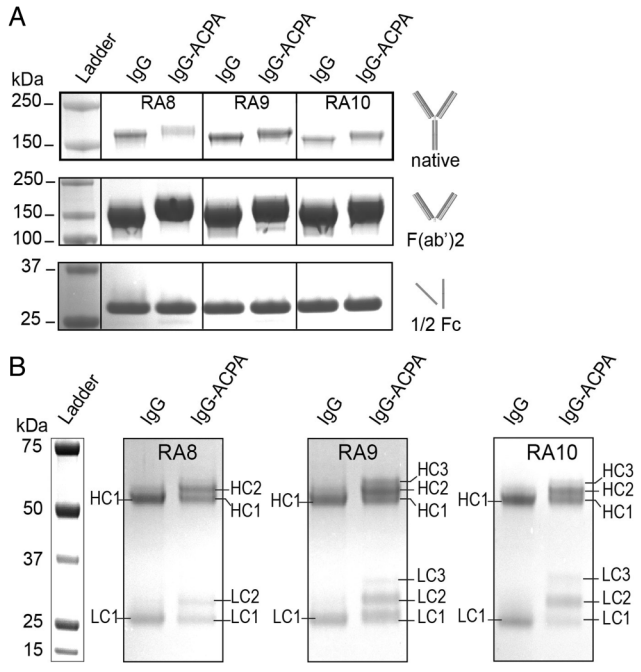

Figure 2 The higher molecular weight of anti-citrullinated protein antibodies (ACPA) is conserved in the F(ab′)$_2$ fragments, as well as in the light chain (LC) and in the heavy chain (HC). (A) SDS-PAGE analysis under non-reducing conditions confirms that native ACPA-IgG or their F(ab′)$_2$ fragments present a 10–20 kDa higher molecular weight relative to other IgG molecules isolated from three rheumatoid arthritis (RA) patients (RA8, 9 and 10). (B) Under reducing conditions, ACPA-IgG exhibit one to three light chains at 26, 30 and 33 kDa (LC1 to LC3) and one to three heavy chains (HC1 to HC3) at 55, 60 and 65 kDa, respectively. In contrast, reduced IgG are mainly represented by one light chain at 26 kDa (LC1) and one heavy chain at 55 kDa (HC1).

of the HC of 'conventional' IgG showed the typical profile of Fc-linked glycans, whereas no glycans were found on the IgG LC (figure 3B).[32 33] In contrast, the additional LC electrophoretic band of ACPA-IgG (LC 2) showed the presence of typical Fab-linked N-glycans.[34] Glycans released from ACPA-IgG HC bands displayed either only the Fc-glycan (HC1) or a mixture of Fc-linked and Fab-linked glycans (HC2 and 3). To further confirm their presence and structure, these glycans were characterised by MS/MS fragmentation (data not shown and see online supplementary figure S2). Based on the SDS-PAGE data presented in figure 2A and online supplementary figure S1 and on the quantification of total, Fc-linked and F(ab′)$_2$-linked N-glycans (data not shown), we estimate that at least 80% of ACPA-IgG molecules contain Fab-glycans.

ACPA F(ab′)$_2$-glycans are linked to the immunoglobulin variable domain

N-glycosidic linkage requires asparagine residues in the protein backbone that are part of a defined consensus sequence (Asn-X-Ser/Thr, where X is not a proline). As no consensus sequence exists in the first constant domain of the IgG HC or LC (CH1/CL1), Fab-linked N-glycans are usually found in the antibody variable region (VH and VL). However, few reports have also described non-consensus glycosylation sites (ie, Ser/Thr-X-Asn) in the CH1 domain of monoclonal IgG and human serum IgG, albeit in very low frequency.[35 36] To determine the exact localisation of ACPA Fab-glycans, electrophoretic bands corresponding to either the entire ACPA molecule, the F(ab′)$_2$-domain, and/or the LC and HC were digested by PNGase F in the presence of $H_2^{18}O$. This reaction allows labelling of the N-glycosylation site through the incorporation of an ^{18}O atom into the Asp-X-Ser/Thr sequence.[25] Following tryptic digestion, ^{18}O-labelled peptides were identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). When applied to the entire ACPA-IgG molecule, this procedure reliably identified the conserved Fc N-glycosylation site at position 297 (Asn297) of the CH2-domain, thereby validating this strategy for identification of N-glycosylation sites (data not shown). LC-MS/MS analysis and peptide sequencing of the ACPA-IgG Fab-region identified six additional N-glycosylation sites in ACPA molecules of seven RA patients. The sequence of these peptides was confirmed by tandem MS of the synthetically prepared counterparts of the proposed tandem mass spectral interpretations. Basic local alignment search tool (BLAST) analysis demonstrated that all N-glycosylation sites contained a N-linked glycosylation consensus sequence located in IgG variable domains (three in HC sequences, two in kappa and one in lambda LC sequences) (table 1). In contrast, neither consensus nor non-consensus glycosylation sites were identified in ACPA-IgG constant domains (CH1 and CL) of the F(ab′)$_2$. In summary, these data show that ACPA-IgG Fab-glycans are linked to conventional N-glycosylation sites within the IgG variable region and indicate that these glycosylation sites have been introduced by somatic hypermutation.

ACPA-IgG Fab-glycans modulate avidity to citrullinated antigens

The predominant function of the antibody variable domain is binding of the cognate antigen. As we could locate the additional N-linked glycans in the variable region of the antibody, we reasoned that it could influence the binding of citrullinated antigens.[31 37–40] Therefore, we next performed ELISA and iSPR experiments to measure the interaction of two ACPA-IgG monoclonal antibodies (mACPA-1 and mACPA-2), naturally glycosylated in their variable region (wild type (WT)),[41] with the CCP2 antigen. These mAbs were also produced in a non-Fab-glycosylated form by mutating the Asn residue of the variable domain N-glycosylation sites back to the germline sequence (non-glycosylated (NG); figure 4A). The presence or absence of Fab-linked N-glycans was controlled by gel electrophoresis of the mACPA WT/NG under reducing conditions (figure 4B). As expected, both mAbs showed binding to CCP2 in ELISA and iSPR (figure 4C, D). When the variable domains were devoid of glycans, mACPA-1 NG exhibits a ~1.9-fold higher binding to CCP2 in ELISA (figure 4C). This result was confirmed by the >2-fold decrease of dissociation constant (KD) (Kd/Ka) measured by iSPR (figure 4D and see online supplementary table S2). Likewise, the absence of the N-linked glycan in the variable domains of mACPA-2 also affected antigen binding. However, in this case, we observed a decrease of binding to CCP2 in ELISA and a >2-fold higher KD in iSPR (figure 4C, D and see online supplementary table S2). Together, these data clearly demonstrate that the variable domain glycosylation of ACPA-IgG can modulate the binding avidity to citrullinated antigens.

DISCUSSION

Our data show a unique feature of ACPA as the vast majority of ACPA molecules in all RA-donor samples analysed thus far contain additional N-linked glycans in their variable domains. Moreover, we have obtained strong indications that the presence of these additional N-linked glycans results from the introduction of N-linked glycosylation sites as a result of somatic

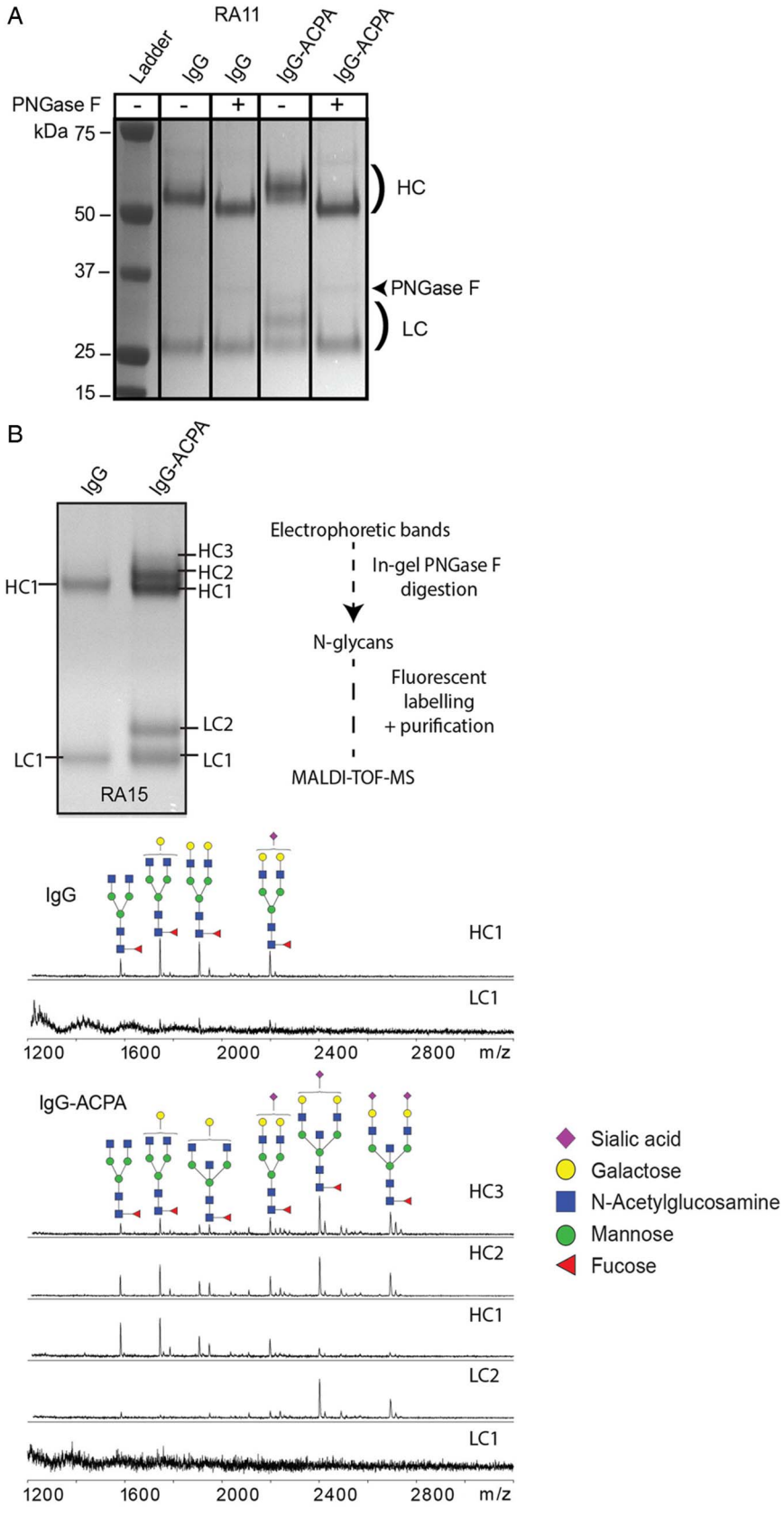

Figure 3 The higher molecular weight of anti-citrullinated protein antibodies (ACPA) results from extensive N-glycosylation. (A) SDS-PAGE of ACPA-IgG and IgG under reducing conditions. Following the release of N-glycans by PNGase F, ACPA-IgG show a similar electrophoretic profile on SDS-PAGE than other IgG molecules, indicating that the additional ACPA-IgG heavy (HC) and light chains (LC) are N-glycosylated. (B) After in-gel PNGase F digestion, the released N-glycans were labelled with the 2-aminobenzoic acid (2-AA) fluorescent tag, purified by hydrophilic interaction chromatography (HILIC) and analysed by matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS). The spectra clearly show the presence of monosialyted and disialylated N-glycans in the additional light (LC2) and heavy chains (HC2 and HC3) of ACPA-IgG, which is not found in conventional IgG. A detailed description of N-glycan structures and their respective masses is depicted in online supplementary figure S2.

hypermutation and that the presence of these unusual sugars can modulate the binding to citrullinated antigens.

The presence of N-linked glycans in the variable domain of human antibodies has been described before. It has been estimated that up to 15–25% of polyclonal human serum IgG of healthy donors can carry Fab-linked N-glycans.[28 34 42] Although further structural analyses will be required to more carefully quantify this percentage in ACPA, our results clearly show that ACPA-IgG are more Fab-glycosylated than other IgG molecules. In contrast, this feature could not be detected for a number of other autoantibodies

Table 1 ACPA-IgG N-glycosylation sites are located within variable regions of the F(ab′)$_2$ fragments

Sample	Peptide sequence	Germline family	Corresponding germline encode sequences	Domain
RA8 F (ab′)$_2$	IN*CSGDALPTR	IGLV3	IGLV3-25*02, IGLV3-25*03, IGLV3-10*01	FR1
RA8 F (ab′)$_2$ RA9 F (ab′)$_2$ RA10 F (ab′)$_2$	GRFN*ISR	IGHV3	IGHV3-53*03, IGHV3-13*02 IGHV3-15*02, IGHV3-21*02, IGHV3-33*02, IGHV3-38*01, IGHV3-48*01, IGHV3-64*01, IGHV3-7*01, IGHV3-9*01, IGHV3-11*01, IGHV3-13*01, IGHV3-19*01, IGHV3-20*01, IGHV3-21*01, IGHV3-74*03, IGHV3-23*02, IGHV3-73*01, IGHV3-30*01, ...	FR3
RA10 F (ab′)$_2$	RPGSSVN*VSCK	IGHV1 IGHV7	IGHV1-69*01, IGHV1-69*04, IGHV1-69*06, IGHV1-46*02, IGHV1-2*01, IGHV1-2*03, IGHV1-45*01, IGHV1-3*01, IGHV1-58*01, IGHV1-3*02, IGHV1-2*02, IGHV1-8*01, IGHV1-24*01, IGHV1-45*02, IGHV1-58*02, IGHV1-69*06, IGHV7-4-1*01, IGHV7-81*01	FR1
RA14 LC	ATIN*CTSSR	IGKV4	IGKV4-1*01	FR1
RA11 HC RA14 HC RA15 HC RA18 HC	GLEWVSSISN*R	IGHV3	IGHV3-21*02, IGHV3-38*01, IGHV3-38*02, IGHV3-d*01, IGHV3-h*01	CDR2
RA15 LC	SN*QSLLFR	IGKV2	IGKV2-28*01, IGKV2-29*01, IGKV2-29*02, IGKV2-40*01	FR1-CDR1

ACPA-IgG tryptic peptide sequences containing N-glycosylation sites identified by LC-MS/MS and their corresponding germline-encoded sequences.
ACPA, anti-citrullinated protein antibodies; HC, heavy chain; LC, light chain; RA, rheumatoid arthritis.

tested. However, due to the limited number of autoantibodies tested so far, we cannot exclude that the unique mechanism of Fab-hyperglycosylation also occurs in other types of autoantibodies, and additional analyses will have to be performed to reveal the full picture of the relevance and consequences of introducing N-linked glycosylation sites during a (chronic) immune response. Nonetheless, our data indicate that the extensive Fab-glycosylation of ACPA-IgG is not a general hallmark of autoantibodies, but rather a specific feature of ACPA or of a particular type of autoimmune response that still needs to be defined. Importantly, the higher molecular weight of ACPA-IgG was also observed in RA patient samples that were collected at the time of diagnosis (see online supplementary table S1). These data indicate that the Fab-glycosylation of ACPA-IgG is already present in early RA patients (disease duration less than 1 year) and suggest that this feature could develop before the onset of RA.

Using high-end mass spectrometry, we found ACPA Fab-portion N-glycosylation sites only in the variable region (table 1). The relatively low number of N-glycosylation sites identified by mass spectrometry is in line with the highly polyclonal nature of ACPA-IgG molecules. Accordingly, protease digestion probably resulted in a high number of variable domain peptides below the detection limit of LC-MS/MS. Therefore, it is likely that the six N-glycosylation sites identified belong to more frequently used immunoglobulin variable domains. In agreement, two of the six N-glycosylation sites were identified in ACPA-IgG of more than one donor (see table 1). More importantly, as all N-glycosylation sites detected by our analysis were not germline-encoded, it is very likely that the high degree of ACPA variable domain glycosylation is the result of extensive somatic hypermutation. The latter finding is intriguing as it suggests that ACPA-producing B cells that have been able to introduce an N-linked glycosylation site during somatic hypermutation have a selective advantage over B cells that failed to do so. Such selective advantage rendered to the B cell could, conceivably, result from the acquired ability of ACPA to interact with lectins that, for example, provide a survival advantage upon cross-linking of the (hyperglycosylated) B cell receptor (BCR). A similar mechanism has been proposed in case of follicular lymphoma B cells where mannose-rich Fab-glycans on cell-surface BCRs create a functional bridge with lectins, thereby providing a survival signal.[43] Thus, the introduction of an N-linked glycosylation site by ACPA-producing B cells could be involved in the 'breach of tolerance' and/or the outgrowth/expansion of these cells.

The thought that somatic hypermutation results in the formation of N-linked glycosylation consensus sequences is also sustained by the high rate of non-synonymous somatic hypermutation detected in ACPA sequences obtained by single cell PCR of synovial fluid B cells and by the generation of Fab-glycosylated ACPA mAbs from peripheral blood, as those used in this study.[41 44] Finally, a recent study in mice further supports this notion by showing that antibodies which bind self-antigens may mask the antigen-binding site with N-glycans and, thereby, relieve the B cell from a continuous BCR signal that is otherwise leading to an 'anergic' state.[40] If this would be the case, it is predicted that Fab-linked glycans modulate binding affinity of ACPA to citrullinated antigens. As we were unable to enzymatically remove the Fab-glycans from isolated polyclonal ACPA without denaturing them, we used monoclonal antibodies that do or do not carry Fab-glycans to study the effect of Fab-glycosylation on antigen-binding affinity. Our ELISA and iSPR experiments showed that the removal of Fab-glycosylation sites alters mACPA binding avidity to the CCP2 antigen. Although we cannot formally exclude that the mutation of amino acids themselves modifies the affinity of monoclonal antibodies for the antigen, our data are in line with the notion that ACPA Fab-glycosylation can influence binding to citrullinated antigens. Interestingly, the modulation of binding depends on the antibody tested and probably also on the nature of the antigen, suggesting that Fab-glycosylation also influences ACPA fine-specificity. In light of this reasoning, it is intriguing that the citrulline-specific immune response, despite signs of extensive isotype switching and somatic hypermutation, has been found to generate antibodies of remarkably low avidity.[15] Moreover,

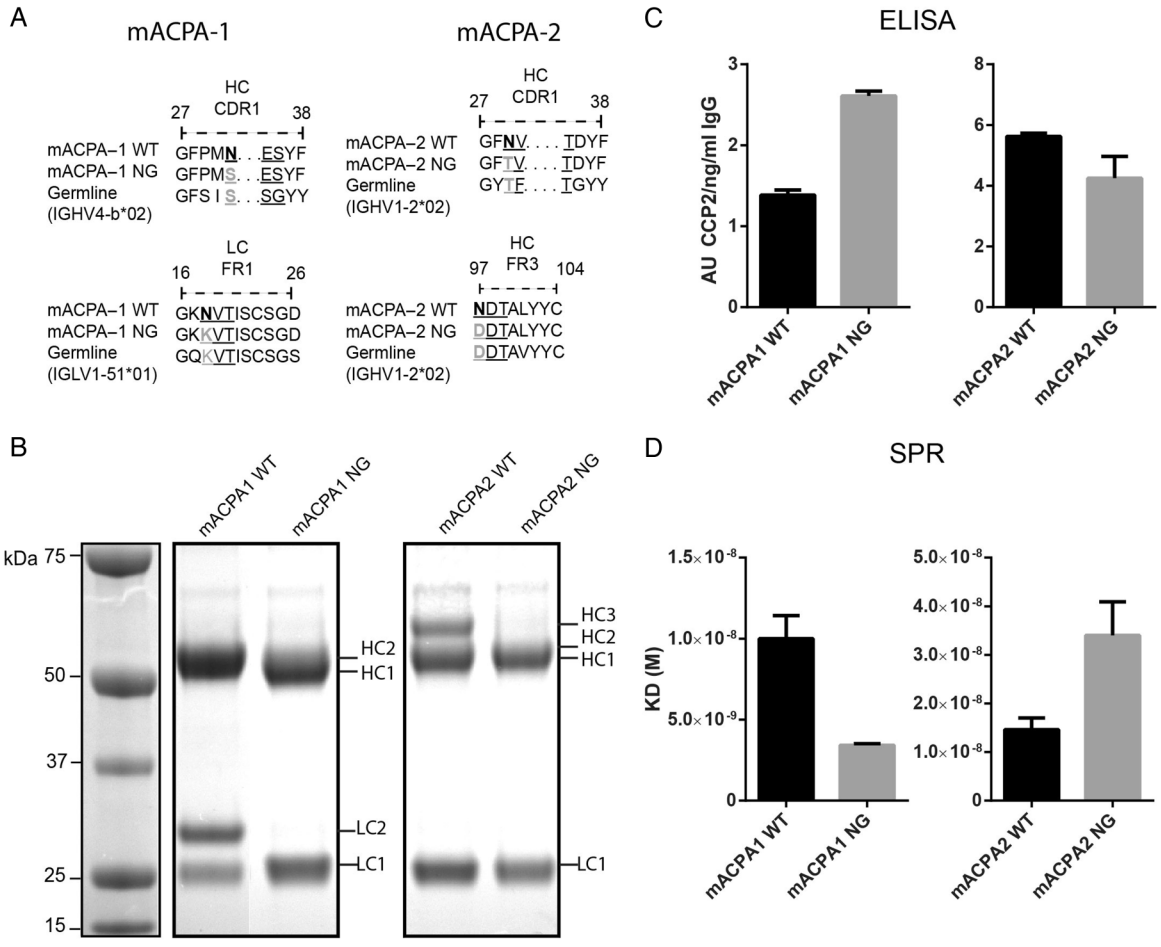

Figure 4 Variable domain glycosylation of anti-citrullinated protein antibodies (ACPA)-IgG modulates binding to CCP2. (A) Partial amino acid sequences of heavy (HC) and light (LC) chains of mACPA-1 and mACPA-2 monoclonal antibodies. Both the HC and the LC chains of wild type (WT) mACPA-1 contain an N-glycosylation site, whereas two sites are present within the HC of mACPA-2 WT. To remove these variable domain N-glycosylation sites and generate non-glycosylated (NG) mACPA, the asparagine residues of the N-glycosylation consensus sequences (Asn-X-Ser/Thr, with X≠Pro) were mutated back to germline-encoded sequences. CDR, complementarity determining region; FR, framework region. (B) Additional heavy (HC2-3) and/or light chains (LC2) observed by SDS-PAGE of mACPA antibodies under reducing conditions confirmed the presence of variable domain N-glycans in these autoantibodies. (C) ELISA experiments (n=2 in quadruplicate) were performed to assess the binding of mACPA-1 and mACPA-2, WT and NG, to CCP-2. ELISA results are reported as arbitrary units (AU) per ng/mL of IgG. (D) Dissociation constant (KD), measured by surface plasmon resonance (n=1 in quadruplicate), representing avidity of the mACPA WT and NG for CCP2. M, molar.

patients harbouring ACPA of lowest avidity display the highest rate of erosive joint destruction.[45] Thus, it is possible that ACPA Fab-glycosylation is responsible for the overall low avidity of the citrulline-specific immune response, which offers a novel perspective of autoantibody function in autoimmunity.

It will be important in future studies to determine whether the presence of an N-linked glycan in the variable domain offers a selective advantage to ACPA-producing B cells or whether other explanations form the basis of ACPA-hyperglycosylation. Likewise, it remains to be determined whether the accumulation of N-glycosylation sites is due to frequent antigen exposure, the diversity of citrullinated antigens or whether it results from other events such as the putative presence of abundant helper activity from CD4+ T cells.[43 46]

In summary, this work demonstrates that structurally different and larger antibodies characterise the RA-specific immune response to citrullinated antigens. This finding points to aberrations in the development of ACPA-specific B cells, reinforces and changes our understanding of basic disease mechanisms in RA and might help to understand how ACPA contribute to RA pathophysiology.

Author affiliations
[1]Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands
[2]Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, the Netherlands
[3]Radboud Institute for Molecular Life Sciences and Institute for Molecules and Materials, Radboud University, Nijmegen, the Netherlands
[4]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands
[5]Netherlands Proteomics Centre, Utrecht, the Netherlands
[6]Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands
[7]Sanquin Research and Landsteiner Laboratory, Academic Medical Center, Amsterdam, the Netherlands
[8]Jan van Breemen Research Institute Reade, Amsterdam, the Netherlands

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Contributors YR, TWJH, LAT, HUS and REMT designed the study. AW, JS, LvT, YR and LAT carried out the gel filtration chromatography and ELISA experiments. The glycosylation analysis was performed by YR with advice from AMD and MW. YR,

PFK, GMCJ and PAvV collected and/or analysed LC-MS/MS data for N-glycosylation site identification. GW and TR provided ACPA monoclonal sequences. AZ and RCH produced ACPA monoclonals. JBCvB and GJMP carried out the SPR measurement and analysis. YR, HUS and REMT interpreted the data and wrote the paper.

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SUPPLEMENTARY MATERIAL

Extensive glycosylation of ACPA-IgG variable domains modulates binding to citrullinated antigens in rheumatoid arthritis

Yoann Rombouts, Annemiek Willemze, Joyce J.B.C van Beers, Jing Shi, Priscilla F. Kerkman, Linda van Toorn, George M.C. Janssen, Arnaud Zaldumbide, Rob C. Hoeben, Ger J.M. Pruijn, André M. Deelder, Gertjan Wolbink, Theo Rispens, Peter A. van Veelen, Tom W.J. Huizinga, Manfred Wuhrer, Leendert A. Trouw, Hans U. Scherer and René E.M. Toes.

SUPPLEMENTARY METHODS

Gel filtration chromatography. Gel filtration chromatography was performed on serum samples of RF seropositive or RF seronegative RA patients with ACPA levels >100 AU/ml using a fast protein liquid chromatography (FPLC) (ÄKTA, GE Healthcare) equipped with a Hiload Superdex 200 (GE Healthcare). Prior to the experiment, the gel filtration column was washed overnight in PBS that was pre-filtered using 0.4μm filters (Millipore). Plasma samples were filtered using Puradisk 30 syringe filter 0.2μm (Millipore) and injected on the FPLC system running at flow rate of 1.0 ml/min. Protein concentration was followed overtime using a UV detector at a wavelength of 280 nm and fractions were collected starting at the beginning of the protein peak. Usually, 50 fractions of 4ml were collected to cover the whole protein peak. From each fraction, 500 μl were used to perform ELISA.

Purification of IgG-ACPA and IgG depleted of ACPA. Prior to purification, plasma samples and synovial fluids were treated respectively with EDTA (1.8 mg/ml) and with hyaluronidase (Sigma Aldrich, 100 μg/ml) from bovine testes type IV (Sigma Aldrich, 1:50). Serum, plasma and synovial fluid samples were then filtered using 0.4 μm filters (Millipore) and applied on fast protein liquid chromatography (ÄKTA, GE Healthcare) equipped with a biotinylated CCP2 arginine control-loaded HiTrap-streptavidin column (GE Healthcare) and a biotinylated CCP2 citrulline-loaded HiTrap-streptavidin column connected in series as described previously [1, 2]. The CCP2 peptides were kindly provided by Dr. J.W. Drijfhout, Department of IHB, LUMC, The Netherlands. ACPA were eluted from the CCP2 citrulline column with 0.1 M glycine-HCl pH 2.5 and directly neutralized with 2 M TRIS. The flow through (FT)

and the fractions eluted from CCP2 citrulline were further purified sequentially on HiTrap protein G and HiTrap protein A columns (GE Healthcare) to obtain IgG depleted of ACPA (isotypes 1, 2 and 4) and IgG-ACPA (isotypes 1, 2 and 4) respectively. In both affinity purifications, the elution of IgG/IgG-ACPA was performed using 0.1 M glycine-HCl pH 2.5 and neutralized with 2 M TRIS. Following protein A purification, fractions containing either purified IgG or purified IgG-ACPA were pooled, concentrated by centrifugal ultrafiltration (Protein Concentrators, 9K MWCO, Pierce Thermo Scientific) and desalted using size exclusion chromatography (Zeba Spin Desalting Columns, 7K MWCO, Pierce Thermo Scientific) according to the manufacturer's instructions. Sample concentrations were measured by nanodrop and by bicinchoninic acid (BCA) Protein Assay Reagent (Pierce Thermo Scientific).

Generation and purification of the F(ab')$_2$/Fc fragments from IgG(-ACPA). In order to generate F(ab')$_2$/ ½ Fc fragments, IgG-ACPA and IgG depleted of ACPA (30 μg of each) were dried under a centrifugal evaporator and digested by adding 200 μL of 50 mM sodium phosphate pH 6.6, 150 mM NaCl containing 33 units (0.5 μL) of ideS (trade name FabRICATOR; Genovis) and incubating overnight at 37°C. The digested antibodies were successively chromatographed on CaptureSelect IgG-Fc (Hu) and CaptureSelect IgG-CH1 affinity beads (BAC) according to the manufacturer. The Fc and F(ab')$_2$ portions were eluted from beads using either 0.1 M glycine-HCl (pH 2.5). Elution fractions were either neutralized with 2 M TRIS and desalted by size exclusion chromatography (Zeba Spin Desalting Columns, 7 kDa MWCO, Pierce Thermo Scientific).

Gel electrophoresis analysis. The purity of (ACPA)-IgG and their Fc/F(ab')2 fragments was checked by SDS-PAGE with NuPAGE Novex 4–12% Bis-Tris gels in an Xcell SureLock Mini-Cell (Invitrogen) according to the manufacturer. Prior to the electrophoresis, samples (2-10 μg) were dried under the centrifugal evaporator and redissolved in 19 μl Laemmli buffer (for non-reducing condition) or 20 μl Laemmli buffer containing 5% β-mercaptoethanol (for reducing condition). Samples were heated for 5 min at 95°C, cooled down at room temperature and finally loaded on the gel. Precision Plus Protein All Blue Standards (BioRad) was used as the molecular weight marker. The gels were run at a constant 200 V for 45 min, stained with Colloidal Blue Staining Kit (Novex, Invitrogen) or SimplyBlue SafeStain (Novex, Invitrogen).

Structural analysis of glycans from (ACPA)-IgG. N-glycans were released from antibody samples by in-gel digestion with PNGase F (Roche). Electrophoresis bands corresponding to IgG-(ACPA) heavy/light chains were excised from gel electrophoresis and transferred to microcentrifuge tubes (Eppendorf). Excised gel pieces were washed several times with 50mM ammonium bicarbonate and acetonitrile (ACN), then reduced in dithiothreitol for 45 min at 60°C and finally alkylated using iodoactemamide for 20 min. After several washing steps with 50 mM ammonium bicarbonate and ACN, gel pieces were incubated overnight at 37°C with 6 μl of PNGase F in 60 μl of 50 mM ammonium bicarbonate to allow the release of N-glycans. Fluorescent labelling of N-glycans was performed as described previously.[3] In short, the solution containing the PNGase F-released N-glycans was

dried under centrifugation, redisolved in 25 µl of MQ water and finally mixed with 12.5 µl of 2-aminobenzoic acid (2-AA; 48 mg/mL) in DMSO with 15% glacial acetic acid and 12.5 µl 2-picoline borane (107 mg/mL) in DMSO. The mixture was incubated for 2 h at 65°C, cooled down at room temperature and diluted to 85% ACN. The 2-AA labelled glycans were purified by hydrophilic interaction liquid chromatography (HILIC) solid phase extraction (SPE) using cotton tips as described previously [4] with some modifications. Briefly, for each sample, 500 µg of cotton were packed into a 200 µl pipette tip and conditioned by pipetting three times 150 µl MQ, followed by 150 µl 85% ACN 0.1% TFA and two times 150 µl 85% ACN. The sample (in 85% ACN) was loaded by pipetting 25 times into the reaction mixture. The tips were washed three times with 20 µl 85% ACN 1% TFA, three times with 150 µl 85% ACN 0.1% TFA and two times 150 µl 85% ACN. The 2-AA labelled glycans were finally eluted from the cotton with 10 µl MQ and identified by Matrix Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) mass spectrometry. Briefly, 2 µl of glycan sample purified by cotton HILIC SPE were spotted on a MTP AnchorChip 800/384 TF MALDI target (Bruker Daltonics), together with 1 µL of 2,5-dihydroxybenzoic acid (DHB) matrix (20 mg/mL in 50% ACN, 50% water). A peptide calibration standard (Bruker Daltonics) was used for external calibration. Analyses were performed in linear negative mode on an UltrafleXtreme MALDI-TOF-MS (Bruker Daltonics) using FlexControl 3.4 software (Bruker Daltonics). For each spectrum, a mass window of *m/z* 1000 to 4000 was used and a minimum of 5000 laser shots were accumulated.

Analysis of N-glycosylation sites. To analyze the sites of glycosylation, in-gel PNGase F digestion was performed using 60 µl of 50mM ammonium bicarbonate dissolved in $H_2{}^{16}O/H_2{}^{18}O$ water (40/60, v/v).[5] After overnight incubation at 37°C, the

solution was removed for glycosylation analysis. The remaining gel pieces were reduced with 10 mM DTT, alkylated with 55 mM and digested with trypsin using the Proteineer DP digestion robot (Bruker Daltonics), equipped with a modified well plate holder to accommodate larger gel pieces. Peptides were extracted from the gel, lyophilized, dissolved in 95/3/0.1 v/v/v water/acetonitril/formic acid and subsequently analyzed by on-line nanoHPLC MS/MS using an 1100 HPLC system (Agilent Technologies), as previously described [6]. Peptides were trapped at 10 µl/min on a 15-mm column (100-µm ID; ReproSil-Pur C18-AQ, 3 µm, Dr. Maisch GmbH) and eluted to a 15 cm column (50-µm ID; ReproSil-Pur C18-AQ, 3 µm) at 150 nL/min. All columns were packed in house. The column was developed with a 30-min gradient from 0 to 50% acetonitrile in 0.1% formic acid. The end of the nanoLC column was drawn to a tip (ID ~5 µm), from which the eluent was sprayed into a 7-tesla LTQ-FT Ultra mass spectrometer (Thermo Electron). The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition. Full scan MS spectra were acquired in the FT-ICR with a resolution of 25,000 at a target value of 3,000,000. The two most intense ions were then isolated for accurate mass measurements by a selected ion monitoring scan in FT-ICR with a resolution of 50,000 at a target accumulation value of 50,000. Selected ions were fragmented in the linear ion trap using collision-induced dissociation at a target value of 10,000. In a post-analysis process, raw data were first converted to peak lists using Bioworks Browser software v 3.2 (Thermo Electron), then submitted to the IPI3.87 human database using Mascot v. 2.2.04 (www.matrixscience.com) for protein identification. Mascot searches were with 2 ppm and 0.5 Da deviation for precursor and fragment mass, respectively, and trypsin as enzyme. Mascot output files were fed into Scaffold v4.0.7 software (www.proteomesoftware.com) for listing

and representation. MsXelerator v2.9.16 (www.msmetrix.com) was used to retrieve the signature pairs of peptides formed by $^{16}O/^{18}O$ labelling during PNGase F. Peak pairs fulfilling a 40/60 ratio and a mass difference of 1.002 ± 0.005 Da (doubly charged ions) were retrieved from the raw data files. Collision-induced dissociation spectra of these retrieved signature pairs were manually inspected and interpreted. The correct identification of all interpreted spectra containing a suspected glycosylation site was confirmed by matching the tandem mass spectra of the gel-eluted peptides with their synthetic counterparts.

Production of ACPA-IgG monoclonal antibodies (mACPA). The mACPA-1 and mACPA-2 used in this study were previously cloned and described by van der Stadt and collaborators.[7] Both antibodies contain two N-glycosylation sites within their variable domains. In order to generate non-glycosylated mACPA, the N-glycosylation sites were removed by mutating the asparagine residue back to the germline-encoded sequence. Heavy and light chain cDNA expressing lentiviral vectors were generated by inserting HC and LC containing PmeI fragments from the previously described pCDNA vectors[7] respectively in pRRL-CMV-bc-GFP and pRRL-CMV-bc-Puro digested with EcoRV. DNA constructs were validated by restriction analysis and sequencing. Third generation self-inactivating lentivirus vectors were produced as described previously.[8] Lentivirus vectors are quantified by antigen capture ELISA measuring HIV p24 levels (ZeptoMetrix Corporation). The infectious titer is derived from the p24 concentration by the conversion that 1ng p24 corresponds to 2500 infectious particles. HEK 293T cells were maintained in DMEM + 10% FCS + Pen/Strep. VL/mACPA-1_{WT}, VL/mACPA-1_{NG} and VL/mACPA-2_{WT} lentiviruses were added to fresh medium supplemented with 8μg/mL polybrene (Sigma), and cells were incubated overnight. Three days post transduction cell cultures were treated

with puromycin (1ug/ml) to select for light chain expressing cells. Stable transductant were then transduced with VH/mACPA-1 WT, VH/mACPA-1 NG and VH/mACPA-2 WT VH/ mACPA-2 NG expressing lentiviruses. Heavy chain expression was determined by fluorescent microscopy and FACS analysis and estimated >90% before use. For each antibody, 50 ml of cell supernatant were harvested and purified on protein A by fast protein liquid chromatography as described above.

Imaging surface plasmon resonance (iSPR). Prior to imaging surface plasmon resonance (iSPR) analysis, mACPA antibodies were purified from 200ml of cell supernatant by FPLC (ÄKTA, GE Healthcare) using a HiTrap protein A column (GE Healthcare) and analyzed by SDS-PAGE. Purified mACPA-IgG were diluted to a final concentration of 18ug/ml in phosphate buffered saline (PBS) with 0.075% Tween 80. Biotinylated CCP2 peptides were immobilized on an iSPR chip containing streptavidin hydrogel linked to a gold layer on a glass surface (Ssens BV).[9] Microarrays were generated using a continuous flow microspotter (CFM; Watsatch). Incubation, washing and regeneration were performed automatically using liquid-handling procedures in a comprehensive SPR imaging system for multiplexing 96 biomolecular interactions (IBISMX96, IBIS Technologies, Enschede, The Netherlands). During the association phase, mACPA were allowed to bind to the immobilized peptides/protein for 40 minutes. Between the association phase and the regeneration phase, the flow cell was rinsed with PBS containing 0.075% Tween 80 for eight minutes. The sensorchip was regenerated with 10 mM glycine-HCl, pH 1.3, for 30 seconds. To obtain quantitative results and to determine affinity constants (kd, ka, and KD), data were analyzed using SPRint software (IBIS Technologies, Enschede, The Netherlands). The reactivity of mACPA (in reactivity units, RU) to CCP2 was deduced by substracting the reactivity to the arginine control variant.

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SUPPLEMENTARY FIGURES AND TABLE

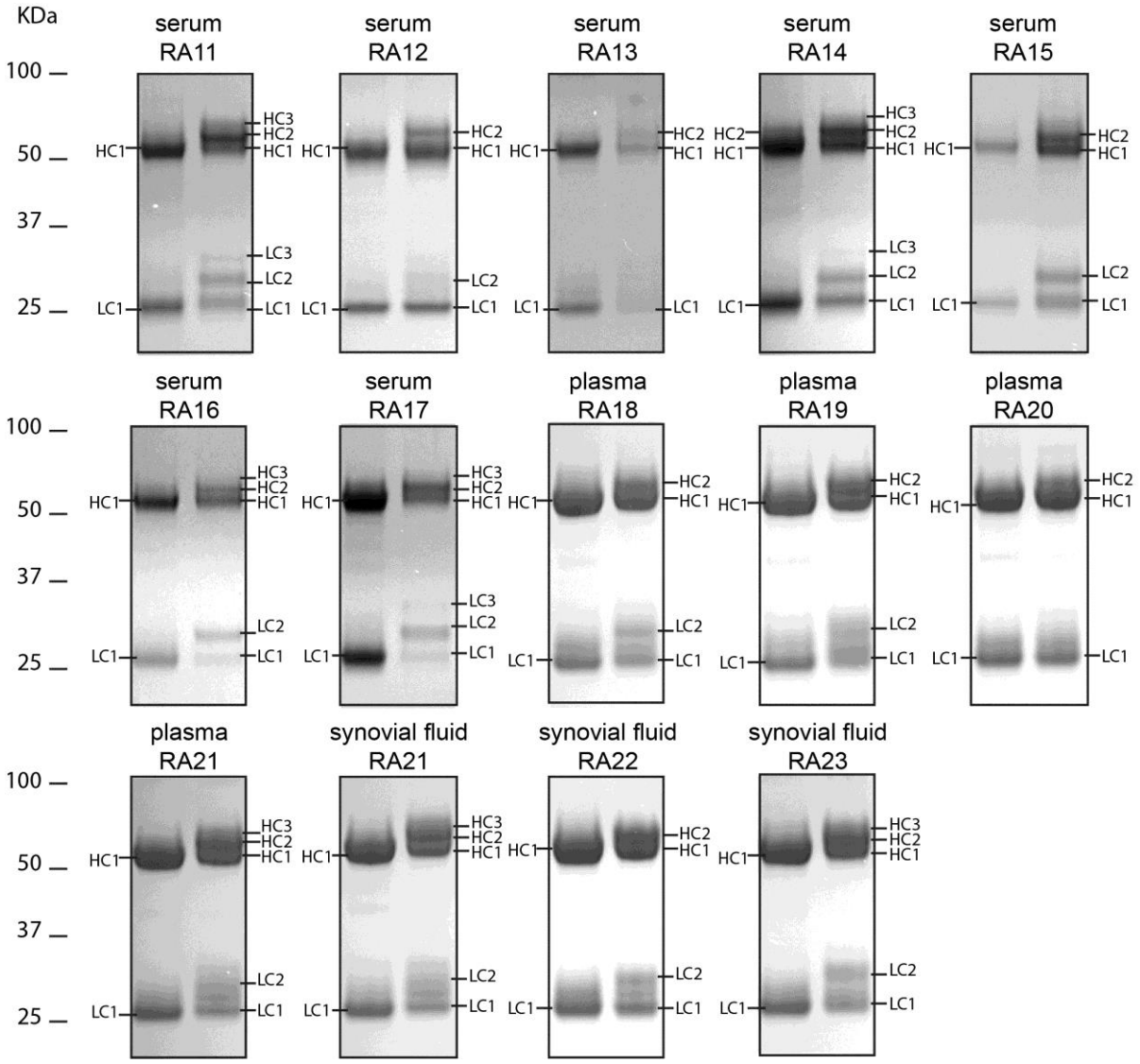

Fig. S1. ACPA-IgG isolated from RA patients exhibit multiple heavy and light chains on gel electrophoresis. As compared to IgG, ACPA-IgG showed several light chains (LC1 to LC3) and heavy chains (HC1 to HC3) on SDS-PAGE under reducing condition.

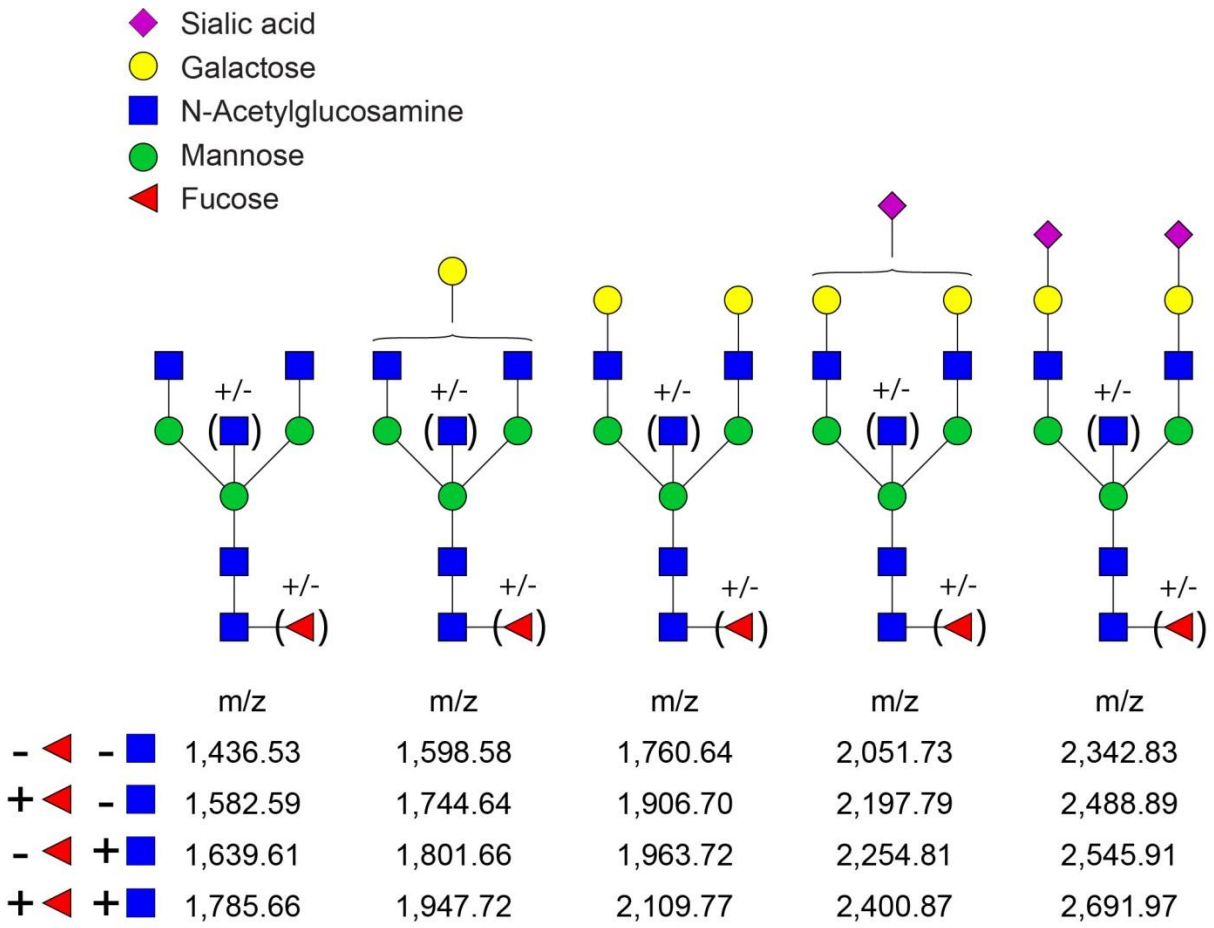

Fig. S2. N-glycans structures of (ACPA)-IgG and their Fc/F(ab')$_2$ fragments identified by MALDI-TOF mass spectrometry. N-glycans of (ACPA)-IgG were released using PNGase F, labelled by 2-AA and purified by HILIC cotton tips. Then, the labelled N-glycans were characterized in negative mode by MALDI-TOF-MS/MS. The *m/z* values correspond to deprotonated species $[M - H]^-$ of the 2-AA labelled N-glycans.

Table S1. Characteristics of the study RA patient samples

Patients	Fluid	Experiment	Sex (f= female and m= male)	Age (yr)	disease duration (yr)	Medication	ACPA serum titre	Disease Activity Score (DAS-44)	Erythrocyte sedimentation rate (mm/h)	Rheumatoid factor detection
RA1	Serum	Gel Filtration	f	45	0,4	mtx 15 mg/wk	392	1,75	2	pos.
RA2	Serum	Gel Filtration	f	64	26	etanercept, mtx 25 mg/wk	nd	1	2	pos.
RA3	Serum	Gel Filtration	f	26	18	tauredon 50 mg/wk im, pred 10 mg	>600	2.42	18	pos.
RA4	Serum	Gel Filtration	f							neg.
RA5	Serum	Gel Filtration	f	46	3	mtx 17.5 mg/wk	184	nd	2	pos.
RA6	Serum	Gel Filtration	f	55	0	none	116	3,23	34	neg.
RA7	Serum	Gel Filtration	m	74	2,5	mtx 20 mg/wk	>600	nd	19	neg.
RA8	Plasma	Purification	f	42	1,5	mtx 20 mg/wk	21011	1,36	6	pos.
RA9	Plasma	Purification	f	74	2,5	tocilizumab 720 mg, pred 10 mg	8691	2,02	8	neg.
RA10	Plasma	Purification	f	59	6	ada 40 mg/2wk, mtx 7.5 mg	20783	2,09	6	pos.
RA11	Serum	Purification	f	62	5	mtx 20 mg/wk	9370	nd	nd	pos.
RA12	Serum	Purification	m	67	5	mtx 25 mg/wk, tauredon	2195	nd	nd	pos.

RA13	**Serum**	**Purification**	**f**	**60**	**1,8**	**mtx 20 mg/wk, sulfasalazine 500 mg 2dd**	**795**	**1,21**	**25**	**pos.**
RA14	**Serum**	**Purification**	**f**	**42**	**12**	**mtx 15 mg/wk**	**>1900**	**1,17**	**6**	**pos.**
RA15	**Serum**	**Purification**	**f**	**47**	**4**	**mtx 15 mg/wk**	**nd**	**nd**	**31**	**pos.**
RA16	**Serum**	**Purification**	**f**	**67**	**2,9**	**mtx 5 mg/wk**	**1278**	**nd**	**nd**	**pos.**
RA17	**Serum**	**Purification**	**f**	**25**	**4,5**	**none**	**2134**	**1,2**	**28**	**pos.**
RA18	**Plasma**	**Purification**	**m**	**34**	**0**	**none**	**37813**	**2,74**	**40**	**pos.**
RA19	**pooled plasma**	**Purification**	**m**	**67**	**27**	**mtx 10 mg/wk, hcq, pred 7,5 mg**	**14641**	**nd**	**19**	**pos.**
						+ tocilizumab	**7399**	**nd**	**nd**	
RA20	**Plasma**	**Purification**	**f**	**57**	**32**	**mtx 12,5 mg/wk**	**12988**	**0,52**	**2**	**pos.**
RA21	**Plasma**	**Purification**	**m**	**55**	**7**	**mtx 20 mg/wk**	**4609**	**nd**	**nd**	**pos.**
RA21	**Synovial fluid**	**Purification**					**2735**			
RA22	**Synovial fluid**	**Purification**	**m**	**56**	**0**	**none**	**18059**	**nd**	**19**	**pos.**
RA23	**Synovial fluid**	**Purification**	**m**	**54**	**14**	**none**	**60896**	**nd**	**94**	**pos.**
n= 23	**Serum (n=14), Plasma (n=7), Synovial fluid (n=3)**		**Female sex, n (%) = 16 (70)**	**median [IQR] = 56 [45-64]**	**median [IQR]= 4 [2-11]**		**median [IQR] = 7399 [1706-16350]**	**median [IQR] = 1,4 [1,2-2,1]**	**median [IQR] = 19 [6-27]**	**RF positivity, n (%) = 19 (83%)**

Table S2. Dissociation constant (KD) and reactivity values of mACPA-1 and mACPA-2 glycosylated (WT) or non-glycosylated (NG) for cyclic citrullinated peptide 2 (CCP2) antigen measured by iSPR. M: molar; RU: resonance units

	Kd (M)	**Reactivity (RU)**
mACPA-1 WT	1,00E-08	2381,5
mACPA-1 NG	3,41E-09	2990,6
mACPA-2 WT	1,46E-08	1984,2
mACPA-2 NG	3,40E-08	2145,0