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Structural Analysis of Variable Domain Glycosylation of Anti-Citrullinated Protein Antibodies in Rheumatoid Arthritis Reveals the Presence of Highly Sialylated Glycans*[S]

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Recently, we showed the unexpectedly high abundance of *N*-linked glycans on the Fab-domain of Anti-Citrullinated Protein Antibodies (ACPA). As *N*-linked glycans can mediate a variety of biological functions, we now aimed at investigating the structural composition of the Fab-glycans of ACPA-IgG to better understand their mediated biological effects. ACPA-IgG and noncitrulline specific (control) IgG from plasma and/or synovial fluid of nine ACPA positive rheumatoid arthritis patients were affinity purified. The *N*-linked glycosylation of total, Fc and F(ab′)$_2$ fragments, as well as heavy and light chains of ACPA-IgG and control IgG were analyzed by UHPLC and MALDI-TOF mass spectrometry. The Fc-glycosylation of ACPA-IgG and IgG was analyzed at the glycopeptide level using LC-MS. The structural analyses revealed that ACPA-IgG molecules contain highly sialylated glycans in their Fab-domain. Importantly, Fab-glycans were estimated to be present on over 90% of ACPA-IgG, which is five times higher than in control IgG isolated from the same patients. This feature was more prominent on ACPA isolated from synovial fluid compared with peripheral blood. These observations provide the first evidence pointing to the ability of ACPA-IgG to mediate novel immunological activities, for example through binding specific lectins via hypersialylated Fab-glycans. ***Molecular & Cellular Proteomics 16: 10.1074/mcp.M116.062919, 278–287, 2017.***

Immunoglobulins are main players of the immune system. IgGs are glycoproteins that contain a conserved glycosylation site located at Asn^{297} present in the Fc-portion (1). From a structural point of view, these Fc-glycans serve as an internal scaffold and are crucial for maintaining the conformation of the Fc tail of the IgG molecule (2). Fc-glycosylation can modulate the interaction with Fcγ-receptors (FcγR) and can be involved in other effector functions, because specific glycoforms can activate the complement pathways (C1q and MBL mediated) and/or interact with lectins (3–5). For instance, absence of core-fucose residues can enhance IgG binding to FcγRIIIa by 50-fold and a lack of core-fucose is responsible for enhanced antibody dependent cellular cytotoxicity (4, 6). Likewise, low content of sialic acid and galactose residues in Fc-glycans have been reported to confer important proinflammatory properties to IgG, as it favors the binding of IgG to activating FcγRs (7).

In addition to Fc-linked *N*-glycans, ~15–25% of IgG molecules in human serum contain *N*-linked glycans present in the Fab-region (8). Fab-glycans can also modulate cellular function and have been implicated in the emergence of lymphoma's such as follicular lymphoma, diffuse large B-cell lymphoma and Burkitt's lymphoma B-cells, presumably through the provision of aberrant Fab-glycosylated B-cell receptor cross-linking via the glycan to lectins (9–12). Recently, we made the intriguing observation that anti-citrullinated protein antibodies (ACPA)[1] isolated from rheumatoid arthritis (RA) patients are extensively Fab-glycosylated (13). ACPA are highly specific for RA and have been implicated in disease pathogenesis, as their presence associates with disease severity and predicts the development of RA in subjects at risk (14, 15). Although it is unknown whether the Fab-glycans on

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[1] The abbreviations used are: ACPA, Anti-Citrullinated Protein Antibodies; Fc, fragment crystallizable; Fab, fragment antigen-binding; FcγR, Fc gamma receptor; C1q, complement component 1 q; MBL, mannose binding lectin; BCR, B cell receptor; CCP, citrullinated cyclic peptide; RA, rheumatoid arthritis; HC, heavy chain; LC, light chain; SF, synovial fluid; IgG, immunoglobulin gamma; UHPLC, ultra-high performance liquid chromatography; HILIC, hydrophilic interaction liquid chromatography; SPE, solid phase extraction; PNGaseF, peptide N glycosidaseF; 2AA, 2-aminobenzoic acid; ACN, acetonitrile; HCl, hydrogen chloride; NaCl, sodium chloride.

IgG molecules can mediate specific functions in normal immune responses, evidence has been obtained supporting the notion that the presence can influence epitope recognition as well as the half-life of antibodies *in vivo* (16–18). To undergo *N*-linked glycosylation, proteins need to express an *N*-linked glycosylation consensus sequence (N-X-S/T, where X ≠ P). Importantly, we previously showed that *N*-linked glycosylation consensus sites in ACPA-IgG were not germ line-encoded but introduced after somatic hypermutation (13). This suggests that ACPA-producing B-cells with an *N*-glycosylation site in the BCR variable domain might have a selective advantage compared with other ACPA-producing B-cells. Finally, Fab glycosylation could also confer biological effector function to ACPA-IgG such as binding to certain lectins expressed on immune cells (8).

The structure of *N*-linked glycans can be highly diverse, and different glycans can interact with different lectins. Therefore, we set out to define the molecular structure of ACPA-IgG Fab-glycans to obtain insight into potential effector functions mediated by these glycans. Here, we report the qualitative and quantitative analysis of *N*-linked sugars present in the Fab-domain of ACPA-IgG using MALDI-TOF, LC-MS, and UHPLC.

MATERIALS AND METHODS

Patient Samples—Plasma ($n = 6$) and synovial fluid ($n = 3$) samples from nine ACPA-positive RA patients were collected at the outpatient clinic of the rheumatology department at Leiden University Medical Center. All RA patients fulfilled the American College of Rheumatology 1987 revised criteria for the classification of RA and gave written informed consent. Permission for conduct of the study was in compliance with the Helsinki Declaration and was approved by the Ethics Review Board at the LUMC. Treatment included disease-modifying anti-rheumatic drugs, biological agents and glucocorticoids. The detailed RA patient characteristics are provided in supplemental Table S1.

Chemicals, Solvents, and Enzymes Used—TFA, SDS, disodium hydrogen phosphate dihydrate, HCl, Glycine, β-mercaptoethanol, acetic acid and NaCl were purchased from Merck (Darmstadt, Germany). Fifty percent sodium hydroxide and Nonidet P-40 substitute, Hyaluronidase from bovine testes type IV, EDTA, 2-aminobenzoic acid, 2-picoline borane complex, ammonium hydroxide, DMSO, 1-hydroxybenzotriazole monohydrate, 2,5-dihydroxybenzoic acid, 2-hydroxy-5-methoxybenzoic acid and formic acid were obtained from Sigma-Aldrich (St Louis, MO). The Laemmli buffer was obtained from Bio Rad (Hercules, CA). Tris and Peptide:N-glycosidase F (PNGase F) were bought from Roche Diagnostics (Mannheim, Germany), 2,5-dihydroxybenzoic acid from Bruker Daltonics (Bremen, Germany) and HPLC SupraGradient ACN from Biosolve (Valkenswaard, Netherlands). MQ (Milli-Q deionized water; R > 18.2 MΩ cm^{-1}; Millipore Q-Gard 2 system, Millipore, Amsterdam, The Netherlands) was used throughout. CaptureSelect anti-IgG Fc affinity matrix and anti-CH1 affinity matrix were bought from Life Technologies (Leiden, The Netherlands). Empty Spin Column with closed screw cap, inserted plug and large 10 μm filters were provided from MoBiTec (Goettingen, Germany). The PBS was obtained from B. Braun (Meslungen, Germany) and the IdeS enzyme (trade name FabRICATOR) from Genovis (Lund, Sweden). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) were provided from Fluorochem, Hadfield, United Kingdom. The CCP2 arginine (control) and CCP2 citrulline peptides were kindly provided by Dr. J.W. Drijfhout, Department of IHB, Leiden University Medical Center (LUMC), and The Netherlands.

Purification of ACPA-IgG and ACPA-depleted IgG from the Plasma and Synovial Fluids of RA Patients—Blood samples were collected in heparin tubes and centrifuged at 3000 rpm for 10 min. Thereafter, the plasma was stored in a 50-ml tube at −20 °C. SF fluids were collected via 5–10 ml syringes by clinicians and immediately centrifuged at 1500 rpm for 10 min. The SF were then stored in 50 ml tubes at −20 °C. Prior to purification, EDTA (1.8 mg/ml) was added to both plasma samples and synovial fluids and the SF was additionally treated with 100 μl hyaluronidase solution (1 mg/ml hyaluronidase (bovine testes type IV) dissolved in 20 mM sodium phosphate, 77 mM NaCl and 1 mg/ml BSA) for 30 min at room temperature. Plasma and SF samples were then centrifuged at 3000 rpm for 10 min and the resulting supernatants were filtered using 0.4 μm filters (Millipore). ACPA-IgG and IgG were purified on fast protein liquid chromatography (ÄKTA, GE Healthcare) as previously described (13). Briefly, samples were loaded on a biotinylated CCP2-arginine-HiTrap-streptavidin column (GE Healthcare) followed by a biotinylated CCP2-citrulline-HiTrap-streptavidin column connected in series. The flow through (FT) and ACPA-eluted fractions were further loaded on a HiTrap protein G and subsequently on a HiTrap protein A column (both from GE Healthcare). The purified IgG and ACPA-IgG of the isotypes 1, 2, and 4 were then concentrated and desalted by size exclusion chromatography (ZebaSpin Desalting Column, 7K MWCO, Pierce Thermo Scientific) according to the manufacturer's instructions.

Generation and Purification of Fc and F(ab′)$_2$ Fragments—ACPA-IgG and ACPA-depleted IgG were specifically cleaved into Fc and F(ab′)$_2$ portions by using the recombinant streptococcal IdeS enzyme. The supplier's protocol was adjusted to simplify the procedure as previously described (13). Briefly, for each sample, 30 μg of (ACPA)-IgG antibodies were dried under centrifugal evaporator and digested by adding 200 μl digestion buffer (50 mM sodium phosphate, 150 mM NaCl, 5 mM EDTA) containing 30U of IdeS followed by incubation at 37 °C for overnight. The Fc portion was then separated from the F(ab′)$_2$ by affinity chromatography on anti-IgG Fc affinity matrix (bead slurry) loaded on a 10 μM filter spin column. The Fc fragments were eluted from beads with 100 mM formic acid and neutralized with 2 M Tris. In order to capture the F(ab′)$_2$ domain, the FT fraction resulting from the Fc purification was purified on anti-IgG-CH1 affinity matrix using a similar protocol as for the anti-IgG Fc affinity matrix. Elution fractions were neutralized with 2 M TRIS and desalted by size exclusion chromatography (Zeba Spin Desalting Columns, 7 kDa MWCO, Pierce Thermo Scientific). Following purification, 6 μg of the purified Fc and F(ab′)$_2$ samples were analyzed for their purity by SDS-PAGE and quantified by bicinchoninic acid Protein Assay Reagent (Pierce Thermo Scientific). For glycan analysis, the samples were dried by vacuum centrifugation.

Glycan Release and Derivatization—The structural analysis was performed on of either the total molecule, F(ab)$_2$ or Fc fragments of the isolated ACPA-IgG and IgG from nine RA patients. In addition, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed of (ACPA)-IgG. *N*-glycans form total molecule, F(ab)$_2$ and Fc fragment of (ACPA)-IgG were released in solution using PNGase F, whereas the heavy and light chain (HC/LC) glycans were obtained following in-gel digestion with PNGase F. Labeling of glycans was performed by mixing the samples (in 25 μl) 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 60 °C, cooled down to room temperature and diluted to 85% ACN prior to purification. The 2-AA labeled glycans were purified by HILIC SPE using cotton tips as described previously with some modifications (19). 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, three times with 150 μl 85% ACN 0.1% TFA and two times 150 μl 85% ACN. The 2-AA labeled glycans were finally eluted from the cotton with 30 μl MQ and identified by MALDI-TOF-MS and UHPLC. Additionally, glycan structures were confirmed through the MALDI-TOF/TOF-MS/MS analysis of *N*-Glycan derivatized by ethyl esterification as previously described with few modifications (20). In brief, 30 μg of ACPA-IgG or IgG were released in solution with PNGase F. Glycan ethylesterification was allowed by mixing 20 μl (out of 25 μl) of the release glycan mixture with 100 μl of the ethylation reagent (250 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, 250 mM 1-hydroxybenzotriazole monohydrate in ethanol) followed by incubation for 1h at 37 °C. Then, 100 μl ACN was added to the samples and the ethylesterified glycans were purified on cotton HILIC-SPE as described above.

UHPLC Analysis and Data Processing—For UHPLC analysis, 5 μl of purified 2-AA labeled *N*-glycan solution were separated and analyzed by HILIC-UHPLC on a Dionex Ultimate 3000 (Thermo Fisher Scientific) equipped with a 1.7 μm 2.1 × 100 mm Acquity UHPLC BEH Glycan column (Waters, Milford, MA) and with a fluorescent detector. Separation was performed at 60 °C with a flow rate of 0.6 ml/min. Two solutions were used for gradient generation, ACN as solution A, and 100 mM ammonium formate pH 4.4 (prepared as formic acid buffered to pH 4.4 by ammonium hydroxide) as solution B. The column was equilibrated by 85% solution A for 0.5 min. The samples were then loaded in 75% A, and excess of fluorescent reagent was eluted from the column by washing with 85% A 47 for 10 min. The separation gradient started at 75% A and decreased linearly to 63% A in 30 min. The column was then flushed at a flow rate of 0.4 ml/min with 40% A for 4 min followed by 10 min of 85% A for re-equilibration. For fluorescent detection, 330 nm was used for excitation and the emission recorded at 420 nm. The resulting chromatograms were analyzed using Chromeleon version 7.1.2.1713 (Thermo Fisher Scientific). The program calculates the area under the curve of the UHPLC chromatograms. Glycan peaks and glycosylation-derived traits were defined (supplemental Fig. S1 and supplemental Table S2) as previously described (21). The percentage of galactosylation (nongalactosylated G_0, monogalactosylated G_1 and digalactosylated G_2), sialylation (nonsialylated N, mono-sialylated S_1 and Disialylated S_2), fucosylation (F) and the frequency of bisecting *N*-acetylglucosamine (GlcNAc, B) residues of IgG were calculated as followed : G0 = GP1+GP2+GP4+GP5+GP6, G1 = GP7+GP8+GP9+GP10+GP11+GP16, G2 = GP12+GP13+GP14+GP15+GP17+GP18+GP19+GP21+GP22+GP23+GP24, *n* = GP1+GP2+GP4+GP5+GP6+GP7+GP8+GP9+GP10+GP11+GP12+GP13+GP14+GP15, S1 = GP16+GP19, S2 = GP21+GP24, F = GP1+GP4+GP6+GP8+GP9+GP10+GP11+GP14+GP15+GP16+GP18+GP19+GP23+GP24 and B = GP6+GP10+GP11+GP13+GP15+GP19+GP22+GP24. SA/Gal was calculated by dividing the total sialylation level (S or SA) by the total galactosylation level (G or Gal).

Mass Spectrometry Analysis and Data Processing—For MALDI-TOF-MS analysis, 2 μl of glycan sample purified by cotton HILIC SPE were mixed on spot with 1 μl of 2,5-dihydroxybenzoic acid matrix (20 mg/ml in 50% ACN, 50% water) on a ground steel MALDI Target (Bruker Daltonics, Bremen, Germany) and allowed to dry at ambient temperature. Measurement was performed in linear negative mode on an UltrafleXtreme MALDI-TOF-MS (Bruker Daltonics) using FlexControl 3.4 software (Bruker Daltonics). A peptide calibration standard (Bruker Daltonics) was used for external calibration. For each spectrum, a mass window of *m/z* 1000 to 4000 was used and a minimum of 5000 laser shots were accumulated. The identification of 2AA-labeled glycans by MALDI-TOF-MS was based on *m/z* values (only if the signal/noise >9) and literature data (supplemental Table S3) (22). The number of assigned and unassigned mass values above threshold of 9 within the linear negative mode spectra of AA-labeled glycan peaks, a median of 95% of all peaks in the intact IgG spectra, 88% of the peaks in the Fc spectra, and 94% of the peaks in the Fab spectra could be linked to glycan structures. With regards to MALDI-TOF/TOF-MS/MS analysis (supplemental Fig. S2 and supplemental Table S4), 3 μl of ethylesterified and purified glycan sample were mixed on spot with 1 μl of superDHB matrix (9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid (5 mg/ml in 50% ACN, 50% water and 1 mM NaOH) on a Bruker AnchorChip plate (800 μm anchor; Bruker Daltonics, Bremen, Germany) and allowed to dry at ambient temperature prior to the measurement. For each spectrum, a mass window of *m/z* 1000 to 4000 was used and a minimum of 2000 laser shots were accumulated. MALDI-TOF/TOF-MS/MS was performed on the 13 highest intensity glycan peaks observed in the spectra of ACPA-IgG and IgG in order to confirm compositions and derive key structural features. MALDI-TOF-MS and -TOF/TOF-MS/MS measurements were performed on a Bruker ultrafleXtreme machine operated in reflectron positive mode. Fragmentation was performed using the Bruker LIFT technology on the most abundant peaks. An internal calibration was performed with *m/z* values indicated in supplemental Table S4. Finally, to analyze the Fc-linked glycosylation of (ACPA)-IgG at the glycopeptide level, antibodies were digested with trypsin and analyzed by LC-MS as described (23, 24). Processing and analysis of the LC-MS data were performed as previously described (23). Briefly, the glycopeptides were identified based on their retention time and *m/z* values. The total intensity of the first three isotopes of every observed analyte charge state was extracted within a window of ±0.06 Da around the theoretical mass and ±20 s around the manually extracted average retention time as described earlier (supplemental Table S5) (25). Double and triple charged analytes were used for the non sialylated species whereas only triple charged values were used for the sialylated glycopeptides (supplemental Table S5).

Statistical Analysis—The statistical analysis was performed using GraphPad Prism 6. A nonparametric paired Wilcoxon test was applied with a significance limit at $p < 0.05$.

RESULTS

Quantification of the N-glycans Expressed by IgG and ACPA-IgG—We have previously demonstrated that ACPA-IgG produced by RA patients are extensively *N*-glycosylated in the variable region if compared with other IgG (auto)antibodies (13). Here we performed a comprehensive quantitative and qualitative analysis of the glycosylation of ACPA-IgG and its fragments and compared it to that of noncitrulline specific IgG (*i.e.* depleted of ACPA hereafter named control IgG). To this end, (ACPA)-IgG were purified by affinity chromatography and their glycans were analyzed by UHPLC, MALDI-TOF-MS and/or LC-MS according to the scheme presented in Fig. 1. Following purification, the purity of (ACPA)-IgG was assessed by SDS-PAGE under reducing conditions (Fig. 2*A*). As expected, control IgG was characterized by two electrophoretic bands corresponding to the heavy and light chains (HC and LC), whereas ACPA-IgG showed several HC and LC bands with higher molecular weights as described previously(13). Released *N*-glycans from both the HC of IgG and the HC1 of

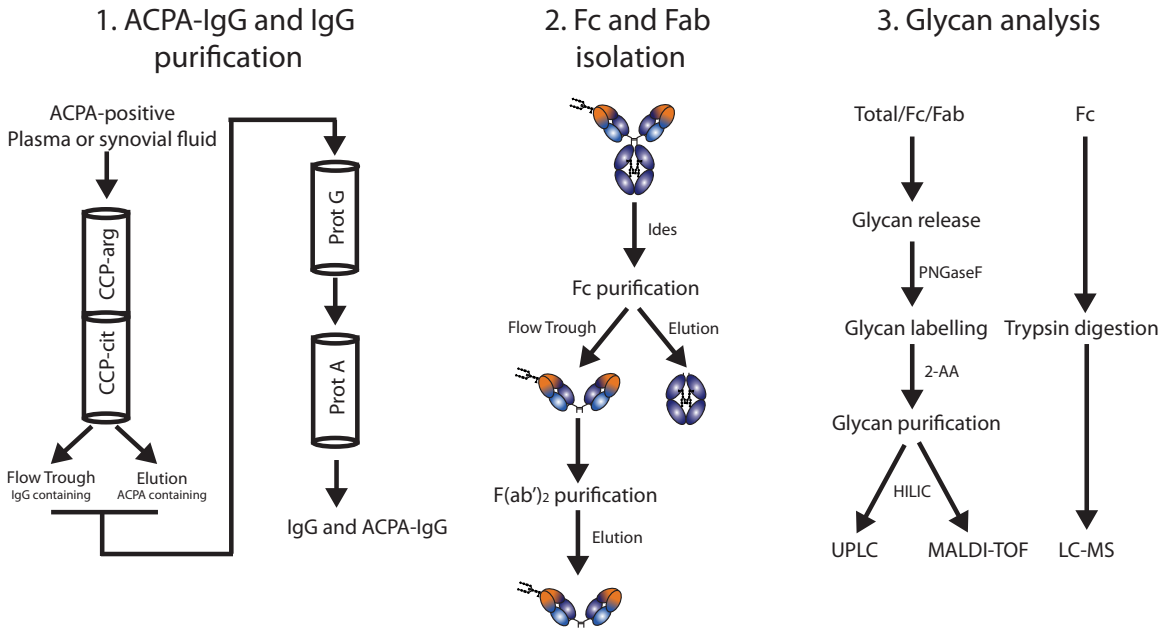

FIG. 1. **Scheme of the purification and analysis of the glycosylation of ACPA-IgG and IgG.** (1) ACPA antibodies were purified by affinity chromatography on CCP2 (citrullinated cyclic peptide (CCP-Cit) or the arginine control (CCP-Arg) followed by Protein G and Protein A capture to obtain ACPA-$IgG_{1,2,4}$ as well as noncitrulline specific $IgG_{1,2,4}$ (depleted of ACPA). (2) (ACPA)-IgG $F(ab')_2$ fragments were generated by digesting purified antibodies with Ides. The resulting Fc part was purified using anti-Fc antibodies, whereas the $F(ab')_2$ fragments were isolated using anti-CH1 domain antibodies. (3) The *N*-glycans of antibodies and fragments were labeled with 2-aminobenzoic acid (2AA) and analyzed by UHPLC and MALDI-TOF-MS whereas the glycopeptides were analyzed by LC-MS.

ACPA-IgG displayed a typical Fc-linked glycan profile in UHPLC (23, 26), whereas no *N*-glycans were detected in the LC of IgG and the LC1 of ACPA-IgG (Fig. 2*B*). In contrast, *N*-glycans released from LC2 of ACPA-IgG showed a different profile, indicating the presence of diantennary glycoforms that were highly sialylated (Fig. 2B and supplemental Table S6). Likewise, the glycosylation profiles derived from HC2 and HC3 of ACPA-IgG showed the presence of a mixture of Fc-glycans but also of additional glycans usually not present in the Fc-domain (Fig. 2B and supplemental Table S6).

Fc-linked and Fab-linked Glycans of IgG (Auto)Antibodies Exhibit Typical Antibody Glycan Patterns—To determine if the glycan pattern detected in the additional HC band of ACPA-IgG, *i.e.* HC2 and HC3, truly reflects the glycosylation of the IgG variable region (27), we investigated the *N*-glycosylation of (ACPA)-IgG and its fragments (Total/Fc/Fab) or glycopeptides (for Fc only) (Fig. 1 and supplemental Fig. S3). We first analyzed and compared the structure of *N*-glycans released from Fc and $F(ab')_2$ fragments of ACPA-IgG and control IgG (from the same donor). The *N*-glycosylation profile derived from (ACPA)-IgG Fc fragments exhibited typical Fc-linked *N*-glycan structures that consisted of diantennary, often core fucosylated complex type species with a variable number of antenna galactose (0 to 2) and sialic acid (0 to 1) residues (Fig. 3*A* and supplemental Fig. S4*A*). Part of the Fc-linked *N*-glycans also contained a bisecting GlcNAc. Of note, a relatively high proportion of agalactosylated glycans (G_0) was observed as previously described (23). The *N*-glycans released from (ACPA)-IgG $F(ab')_2$ fragments consisted of highly galactosylated and sialylated diantennary glycoforms, that may carry bisecting GlcNAc and/or a core fucose (Fig. 3*B* and supplemental Fig. 4*B*). Together, the results demonstrate that the *N*-glycan species attached to the Fc and Fab fragments of IgG (auto)antibody differ with a striking presence of highly sialylated glycan species in the glycans linked to the Fab-domain of ACPA-IgG.

The Fab-linked Glycosylation Pattern of ACPA-IgG Differs from the Pattern on "Conventional" IgG—We have previously shown that Fc-linked *N*-glycans of ACPA-IgG isolated from patients present a more pronounced reduction in the level of galactosylation and sialylation but an increased degree of core fucosylation than those of other IgG molecules (23, 26). In agreement, the Fc-glycans of ACPA-IgG purified in this study exhibit a lower level of sialylation (S 12% [IQR9–16%] for ACPA-IgG *versus* 16% [IQR13–17.5%] for control IgG) as well as a higher frequency of core fucosylation in comparison with that of control IgG (F 99.3% [IQR98.7–99.7%] for ACPA-IgG *versus* 91.8% [IQR90.3–99.7%]) IgG (supplemental Fig. S5, S6 and supplemental Table S7, S8, and S9). In addition, our data revealed important differences between the Fab-linked *N*-glycan profile of ACPA-IgG and that of control IgG (Fig. 4 and supplemental Table S10 and S11). Especially, ACPA-IgG Fab *N*-glycans displayed a high frequency of di-galactosylated species (G2; 73% [IQR69.5–80%] for IgG *versus* 84% [IQR74–87%] for ACPA-IgG) and di-sialylated species (S2; 27% [IQR19–30%] for IgG *versus* 44% [IQR34–

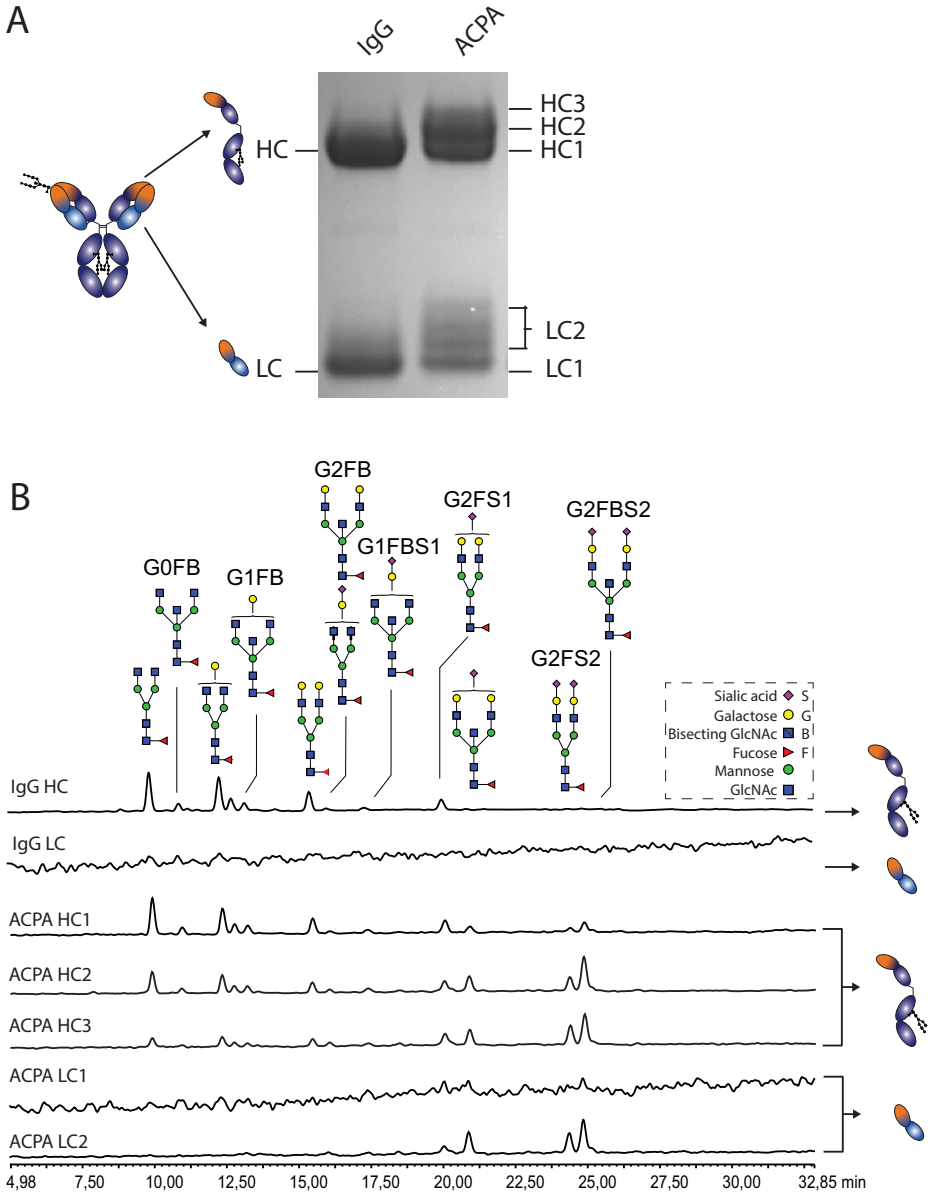

FIG. 2. **The glycosylation of heavy chain (HC) and light chain (LC) derived from ACPA-IgG and IgG isolated from RA patients.** *A*, SDS-PAGE of ACPA-IgG and IgG under reducing condition. Compared with control-IgG exhibiting one HC and one LC, ACPA-IgG showed multiple HC (HC1 to HC3) and LC bands (LC to LC2) because of *N*-linked glycosylation (13). *B*, UHPLC chromatograms of the *N*-glycans extracted from the different electrophoretic bands of a representative donor.

48.5%] for ACPA-IgG), as also exemplified by an increase in the ratio of sialic acid per galactose (SA/Gal; 36% [IQR33–37%] *versus* 30% [IQR27–31.5%] for ACPA-IgG and IgG). In addition, we found higher levels of core fucose and bisecting GlcNAc residues in 7 out of 9 samples. In general, stronger glycan differences were observed between the glycan structures derived from the Fab domain of ACPA-IgG and control IgG than between the glycans from the Fc portions.

ACPA-IgG Exhibit a Higher Level of Fab Glycosylation—We next quantified the amount of Fab glycosylation present on ACPA-IgG and IgG depleted from ACPA. To estimate the level of Fab glycosylation, glycans were released from ACPA-IgG and ACPA-depleted IgG, characterized by MALDI-TOF-MS and their relative abundance was measured by UHPLC. Whereas the glycan profile of total IgG was dominated by Fc-linked *N*-glycans (G0F, G1F and G2F), the total glycan profile of ACPA-IgG exhibited a large quantity of Fab-linked *N*-glycan (G2FBS1, G2FS2 and G2FBS2) (Fig. 5*A* and supplemental Fig. S7). Importantly, the identification of a number of these glycoforms specific for either the Fc- or the $F(ab')_2$-fragment, and the quantification of these glycoforms released from the entire antibody molecule, enabled us to determine the overall frequency of Fab glycans on either ACPA-IgG or ACPA-depleted IgG (Fig. 5*B*). All ACPA-IgG samples ($n = 9$) exhibited an increased frequency of Fab glycosylation compared with control IgG. The median Fab-glycosylation level of IgG depleted of ACPA was estimated at 17% [IQR12–26%], with large differences between donors. In contrast, the median Fab glycosylation of ACPA-IgG reached 93% [IQR77–123%]. Together, these data indicate that the median Fab

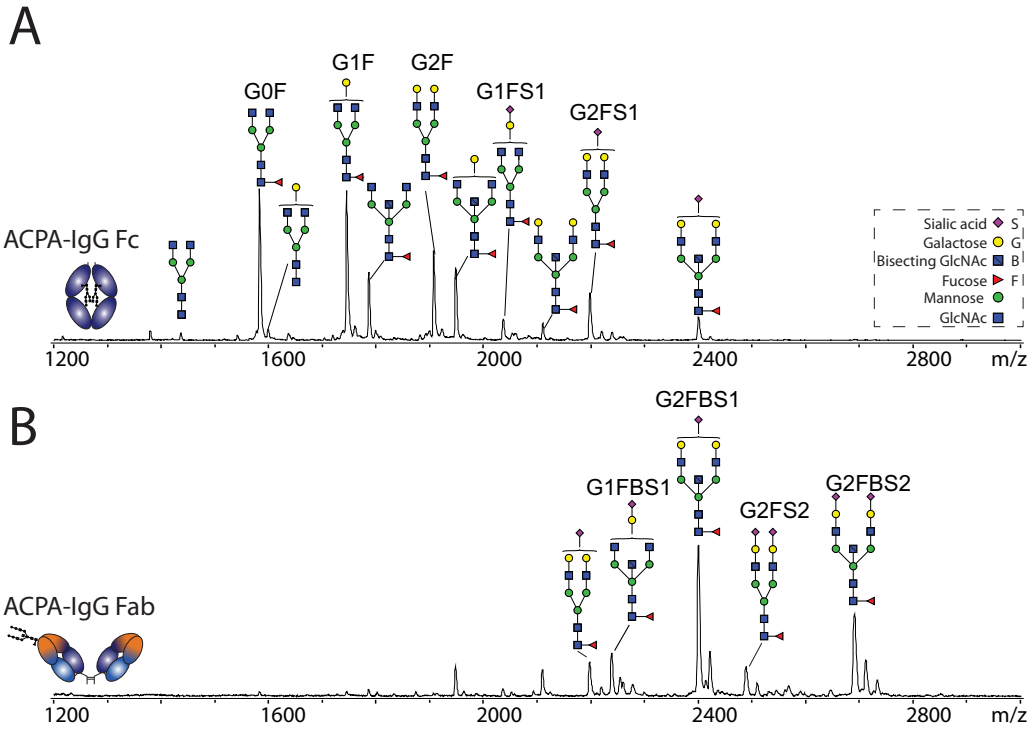

FIG. 3. **ACPA-IgG is differentially glycosylated in the Fc compared with the Fab glycosylation.** MALDI-TOF spectra of the (*A*) Fc and (*B*) F(ab′)$_2$ fragments of ACPA-IgG from a representative donor.

glycosylation of ACPA-IgG is 5 times higher than that of control IgG.

(ACPA)-IgG Derived from Plasma and Synovial Fluid Display Different Fab Glycosylation Profiles—We previously demonstrated that ACPA-IgG derived from the synovial fluid display a more proinflammatory Fc glycosylation profile than ACPA-IgG purified from serum (28). Given this observation, we hypothesized that differences may also occur in the Fab-linked glycan structures and/or Fab-glycosylation levels of ACPA-IgG and control IgG. As compared with the plasma ACPA-IgG ($n = 6$) Fab-linked glycans, the composition of SF derived ACPA-IgG ($n = 3$) Fab glycans exhibited a trend toward lower levels of galactosylation, sialylation, and bisecting GlcNAc. A similar trend was observed for the glycan profile of SF control IgG compared with plasma IgG (supplemental Fig. S8). We next quantified the level of Fab-glycosylation of plasma (ACPA)-IgG and their counterparts from the SF. As shown in Fig. 5*C*, a significantly higher level of Fab glycosylation was found in ACPA-IgG from the SF as compared with plasma ACPA-IgG (138% *versus* 80%). Of note, such a difference was not observed for control IgG (20% *versus* 20%). Together, these observations indicate in quantitative terms that the level of Fab-glycosylation is even more pronounced on ACPA-IgG form SF as compared with ACPA-IgG from blood.

DISCUSSION

ACPA-IgG is a highly relevant prognostic and diagnostic biomarker for RA. ACPA-positive RA is characterized by a high rate of joint erosions and a low chance to achieve remission when left untreated (15). Earlier, we and others reported that the galactosylation and sialylation levels of Fc-glycans from ACPA-IgG is lower compared with control IgG, and this is more pronounced in ACPA-IgG isolated from SF (28, 29). In addition, we observed that the changes of ACPA-IgG Fc-glycosylation already occur a few months before of the diagnosis of RA (23).

Next to Fc-glycosylation, we have recently reported that ACPA-IgG are extensively glycosylated in their variable domain; a feature that may modulate the function of ACPA-IgG and that could be involved in the pathophysiology of RA (8). So far, however, a detailed structural analysis of ACPA Fab-linked glycans was lacking (13). We now provide a qualitative and quantitative characterization of Fab-linked *N*-glycosylation of ACPA-IgG and control IgG isolated from plasma and SF of RA patients to better understand potential functional consequences of ACPA Fab glycosylation.

We found that the glycans attached to the Fab-portion of ACPA-IgG consist of diantennary complex type *N*-glycans with high sialylation and galactosylation contrasting with the composition of *N*-glycans linked to the Fc-part of (auto)-antibodies derived from RA patients (Fig. 3*A*). Interestingly, the Fab-glycosylation pattern of ACPA-IgG showed a significantly higher frequency of galactose, sialic acid and fucose residues as compared with that of control IgG depleted of ACPA. These high galactosylation and sialylation levels of the Fab-linked glycans are in clear contrast to the lower level of

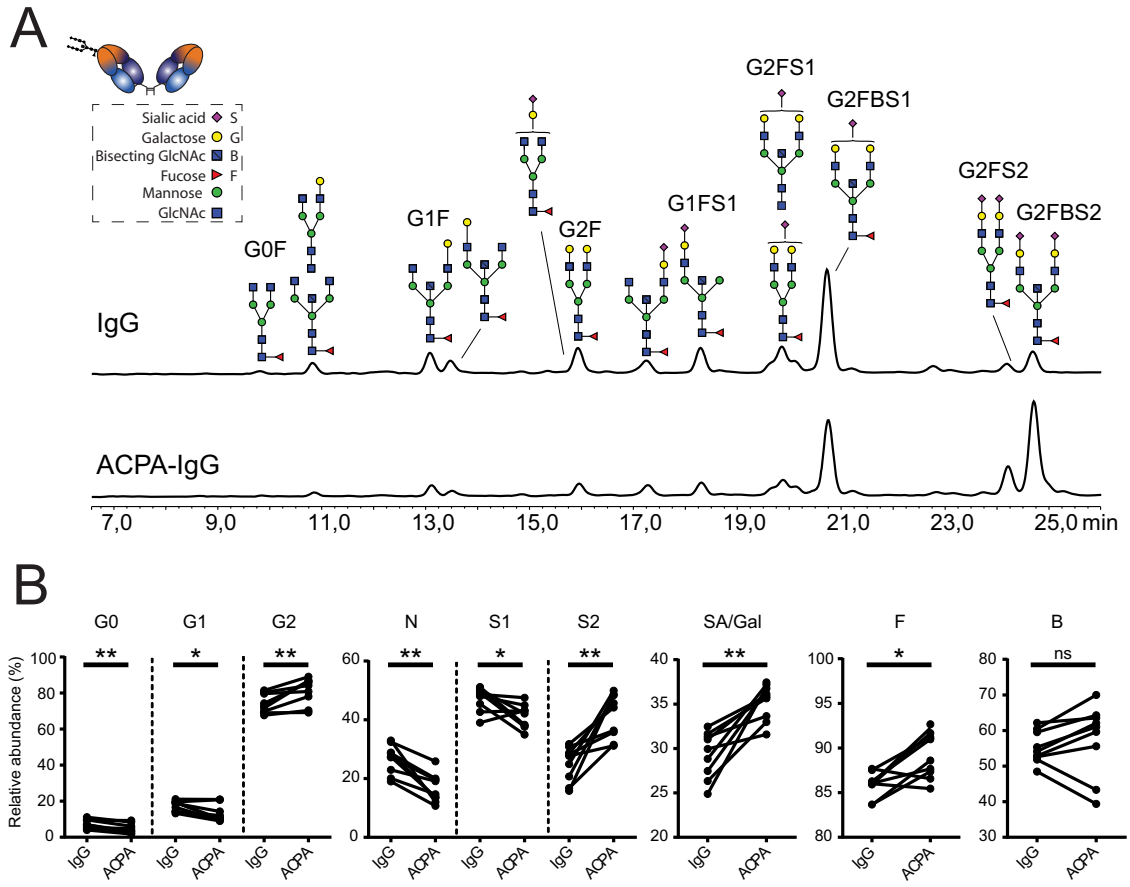

FIG. 4. **The Fab-linked glycosylation patterns differ between ACPA-IgG and noncitrulline specific IgG isolated from RA patients.** *A*, UHPLC chromatograms of ACPA-IgG and IgG F(ab')$_2$ glycans of a representative RA patient. *B*, Differences in glycan-derived traits of ACPA-IgG and IgG Fab glycosylation represented in the relative abundance of galactosylation, sialylation, fucosylation and bisection (supplemental Table S9 and S10).

galactosylation and sialylation previously detected in the Fc-part of ACPA-IgG (23, 28, 29). Therefore, our data show that the changes in antibody glycosylation occurring during RA are not only (auto)antibody-specific, but also site-specific.

The reason why the Fc-glycan composition differs from the Fab-glycan composition is not known, but is conceivably a consequence of the accessibility of these glycans to glycosyltransferases present in the (trans)Golgi of B-cells. Likewise, as we found differences in glycan composition between ACPA-IgG and control IgG, it is likely that also the composition of the glycosyltransferases/glycosidases in different B-cell populations is differently regulated. How Ig glycosylation is regulated is currently not well defined. We have previously shown that the cytokine-milieu in which B-cells produce antibodies influences the Fc-glycosylation profile, indicating environmental control of Ig-glycosylation possibly explaining the glycosylation differences observed between ACPA-IgG and control IgG, as well as well as between serum/plasma antibodies and their synovial fluid counterparts (30).

Through a detailed characterization of the glycosylation of (ACPA)-IgG by UHPLC and mass spectrometry, we determined for the first time the percentage of Fab-glycosylation in ACPA-IgG and control IgG (ACPA-depleted) isolated from RA patients. It has been estimated that up to 10–25% of healthy donor IgG molecules carry Fab-linked *N*-glycans (8). In line with this, our current data indicate that plasma and SF IgG (depleted of ACPA) of RA patients exhibit a median level of Fab-glycosylation of around 17%, albeit with variation between donors. In contrast, the median Fab-glycosylation level of ACPA-IgG was calculated to be 5 times higher and reached a median level of 93%. In some patients, this percentage was above 100%, which strongly suggests that ACPA-IgG can exhibit multiple glycosylation sites in their variable regions. Accordingly, each of the two ACPA-IgG monoclonal antibodies recently cloned by Rispens and coworkers contain two *N*-glycosylation sites per Fab portion, and all sites appear to be occupied by glycans (13, 31).

Importantly, the calculated percentage of ACPA-IgG Fab-glycosylation is probably underestimated when the UHPLC profile of the Fc was used. Indeed, although almost no disialylated glycans were observed in the ACPA-IgG Fc-glycopeptide data obtained by LC-MS, the UHPLC profiles of glycans

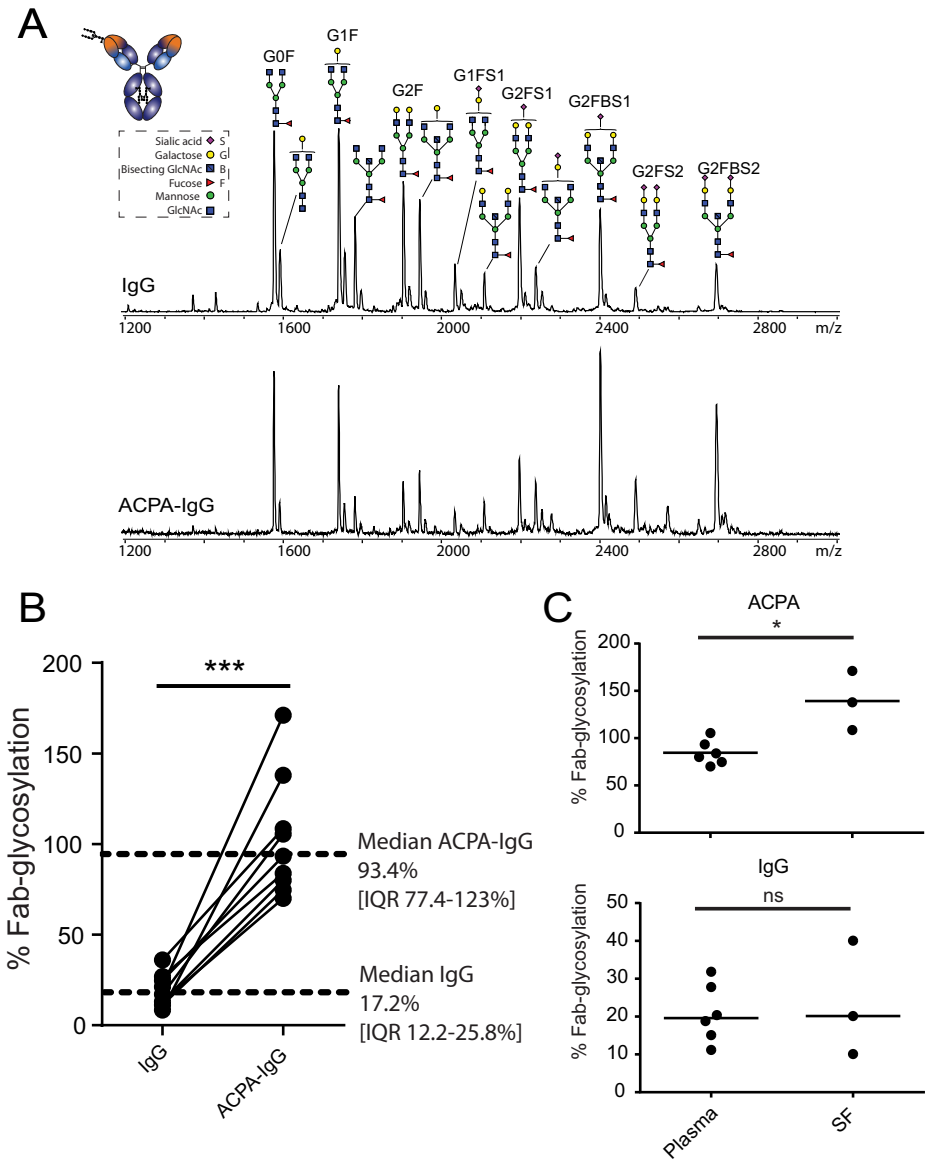

FIG. 5. **ACPA-IgG are highly Fab-glycosylated compared with noncitrulline specific IgG.** *A*, MALDI-TOF-MS spectra of ACPA-IgG and IgG. *B*, Comparison of ACPA-IgG and IgG Fab-glycosylation levels derived from UHPLC and LC-MS data. *C*, Comparison of the Fab glycosylation of ACPA-IgG or noncitrulline specific IgG from synovial fluid ($n = 3$) and plasma ($n = 6$).

released from the Fc part of ACPA-IgG showed higher proportions of disialylated species, especially G2FS2 and G2FNS2, which belonged to the Fab domain (data not shown). Therefore, the presence of these disialylated species, albeit at low level, in the UHPLC-based Fc-glycosylation profile of ACPA-IgG is very likely because of a low level of missed cleavage by IdeS. Therefore, the LC-MS data was used for the calculation of the % Fab-glycosylation.

As recently reviewed by us, several different functions have been allocated to antibody Fab-glycosylation (8). The extensive presence of Fab-glycans may increase the serum half-life of ACPA-IgG as previously reported for some monoclonal antibodies. Although further experiments are required to deepen and validate this hypothesis, the potential protective effect of the Fab-glycosylation on ACPA-IgG clearance could have important implication for the understanding of RA pathophysiology and may be one of the reasons of the relatively high levels of these autoantibodies in RA patients (32).

In addition, through the binding of Fab-glycans to lectins expressed on immune cells, ACPA-IgG may have the capacity to modulate cellular functions as demonstrated for other Fab-glycosylated antibodies (8). It is clear across studies that the Fab-glycans can modulate the binding of antibody to antigens. Indeed, we have previously demonstrated that Fab-glycosylation can either decrease or increase the binding of ACPA-IgG to the synthetic cyclic citrullinated peptide (13). We are currently investigating this aspect in more depth to obtain a broader understanding of this functional aspect by examining more antigens as well as by analyzing the influence of different patterns of Fab-glycosylation to antigen binding.

With regard to B-cells, it has been proposed that *N*-glycans linked to the Fab-portion of the BCR can be recognized by

lectins, thereby stimulating B-cell survival. Specifically, it has been shown that even in the absence of antigen, follicular lymphoma B-cells are activated through the binding of their high-mannose type glycan containing BCR to lectins (DC-SIGN, mannose receptor) at the surface of macrophages and/or dendritic cells (11, 12, 33). A similar biological mechanism may apply to ACPA-producing B-cells. The latter notion would provide an explanation for our observation that the ACPA response only undergoes limited avidity maturation in time, conceivably because ACPA-producing B-cells are not selected for higher affinity for the antigen but rather for their ability to receive Fab-glycan lectin-mediated prosurvival signals.

In summary, our study pinpointed, for the first time, that the pattern and level of Fab-glycosylation differs markedly between ACPA-IgG and noncitrulline specific IgG as well as between ACPA isolated from the SF or blood. Given that Fab-glycans conceivably impact antibody properties/activities as well as the development and survival of B-cells (9–12), our structural findings are of relevance and importance to better understand the biological capabilities of ACPA and ACPA-producing B-cells.

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