# Fc gamma receptor binding profile of anti-citrullinated protein antibodies in immune complexes suggests a role for FcγRI in the pathogenesis of synovial inflammation

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## Abstract

### Objective

*Anti-citrullinated protein antibodies (ACPA) are highly specific for rheumatoid arthritis (RA). Here, we studied binding of ACPA-IgG immune complexes (IC) to individual Fc gamma receptors (FcγR) to identify potential effector mechanisms by which ACPA could contribute to RA pathogenesis.*

### Methods

*ACPA-IgG1 and control IgG1(IgG1 depleted of ACPA-IgG1) were isolated from plasma and synovial fluid (SF) of RA patients by affinity chromatography using CCP2 peptides. Subsequently, IC were generated using fluorescently labelled $F(ab')_2$ fragments against the $F(ab')_2$ region of IgG, or by using citrullinated fibrinogen. IC were incubated with FcγR-transfected CHO cell lines or neutrophils from healthy donors. FcγR binding of IC was analysed by flow cytometry in the presence or absence of specific blocking antibodies.*

### Results

*ACPA-IgG1 IC predominantly bound to FcγRI and FcγRIIIA on FcγR-transfected CHO cell lines, while much lower binding was observed to FcγRIIA and FcγRIIB. ACPA-IgG1 IC showed reduced binding to FcγRIIIA compared to control IgG1 IC, in line with enhanced ACPA-IgG1 Fc core-fucosylation. Neutrophils activated in vitro to induce de novo expression of FcγRI showed binding of ACPA-IgG IC, and blocking studies revealed that almost 30% of ACPA-IgG IC binding to activated neutrophils was mediated by FcγRI.*

### Conclusion

*Our studies show that ACPA-IgG1 IC bind predominately to activating FcγRI and FcγRIIIA, and highlight FcγRI expressed by activated neutrophils as relevant receptor for these IC. As neutrophils isolated from SF exhibit an activated state and express FcγRI in the synovial compartment, this IC-binding could contribute to driving disease pathogenesis in RA.*

**Key words**

rheumatoid arthritis, ACPA, immune complexes, Fc gamma receptors, FcγRI, IgG, neutrophils

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## Introduction

In rheumatoid arthritis (RA), a chronic autoimmune disease defined by joint destruction and persistent inflammation of synovium, anti-citrullinated protein antibodies (ACPA) are believed to be involved in disease pathogenesis. ACPA serve as predictive biomarkers, as ACPA are frequently present in serum before the onset of disease (1). Moreover, ACPA are highly specific for RA and constitute risk factors for severe disease (2). Of note, the vast majority of ACPA in the circulation and in synovial fluid (SF) are secreted as IgG molecules, and citrullinated antigens have been detected in rheumatoid synovium (3, 4). Therefore, it is likely that ACPA-IgG immune complexes (IC) interact with immune cells in the context of synovial inflammation. The role of ACPA in RA pathogenesis has become an important topic of investigation but exact effector mechanisms of these autoantibodies are still incompletely understood.

Antibody effector functions are mainly mediated via the antibody constant (Fc) region, which can bind to specific Fc receptors (FcR) expressed by immune cells. Fc gamma receptors (FcγR) interact with the IgG Fc tail and are categorised in the high-affinity receptor FcγRI and low-affinity receptors FcγRII and FcγRIII. FcγRI is considered to be the only receptor capable of binding monomeric IgG and is thought to bind IC only upon *de novo* surface expression or cellular activation (5). In contrast, FcγRII and FcγRIII do not bind monomeric IgG but only complexed IgG. Activating FcγR mediate cellular activation via immunoreceptor tyrosine-based activation motifs (ITAM), whereas the only inhibitory FcγR, FcγRIIB, has an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic tail, which can counteract signalling cascades of activating receptors (6, 7). Additional diversity in the FcγR repertoire is generated by polymorphisms for FcγRIIA, FcγRIIIA and FcγRIIIB. An amino acid substitution of arginine (R) to histidine (H) at position 131 in FcγRIIA results in increased binding of IgG2 to this receptor (8). FcγRIIIA polymorphism V158 (also known as V176F) leads to enhanced binding affinity for all IgG subclasses and is more frequently found in European RA patients compared to FcγRIIIA (F158) (8-10). Factors determining the binding specificity of monomeric or complexed antibodies for the different FcγR include the type and level of FcR expressed, the IgG subclass, the size of the antibody-antigen IC, glycosylation of the FcR and, more importantly, the Fc glycosylation profile of the antibody (8, 11-13). Importantly, immune cells differentially co-express activating and inhibitory FcγR, which is thought to create a balanced threshold for cellular triggering. Given the complexity of this system, it is important to study binding characteristics of antibodies to various FcγR individually to understand specific antibody-mediated effector mechanisms.

Interestingly, recent work has provided experimental support for the involvement of ACPA IC-mediated effector functions in RA pathogenesis. Several studies investigated the effect of ACPA-containing IC on cytokine secretion by monocytes and macrophages *in vitro* as synovial macrophages are potent producers of tumour necrosis factor α (TNF-α). Indeed, plate-bound ACPA-containing IC can induce TNF-α secretion by macrophages and PBMCs (14-17). These and additional murine studies have pointed to FcγRIIA as a relevant mediator of ACPA-IC triggered cytokine secretion by macrophages (18). However, in the inflamed synovium of RA patients distinct immune cells are present, which express different combinations and levels of FcγR that can balance ACPA-IC mediated effects. To dissect this complexity, we aimed to study ACPA-IC binding characteristics to individual FcγR using an experimental setting which allows to control for the expression level of each individual FcγR. To translate our findings in a more representative setting, we subsequently studied binding of ACPA-IC to FcγR on resting and activated neutrophils as neutrophils represent the main cell type present in SF of RA patients.

## Methods

*Patients and healthy individuals*

Four peripheral blood and four SF samples were obtained from ACPA-positive

**Table I.** Patient characteristics of patients included in the present study.

| Patient | Age | Sex | Material (serum/synovial fluid) | Erosive disease (x-ray) | Time since diagnosis (yrs) | ESR (mm) | Activity (DAS 44) | Treatment at the time of sampling |
|---|---|---|---|---|---|---|---|---|
| RA1 | 56 | M | Synovial fluid | no | 0 | 19 | 1.26 | none |
| RA2 | 77 | F | Serum | yes | 42 | 25 | n.d. | Mtx 5 mg/week, Prednisolon 5 mg/d, Rituximab |
| RA3 | 49 | F | Serum | yes | 21 | 9 | 1.55 | Mtx 5 mg/week, Adalimumab 40 mg eow |
| RA4 | 69 | F | Serum and synovial fluid | yes | 23 | 9 | 1.68 | Etanercept 50 mg eow |
| RA5 | 57 | F | Serum | yes | 44 | 6 | 0.88 | Mtx 10 mg/week |

Disease activity was assessed using DAS scores evaluating 44 joints and using three variables (ESR, tender and swollen joint count) [37]. The scores can be interpreted as low (DAS ≤ 2.4), moderate (2.4 < DAS ≤ 3.7), or high (DAS > 3.7) disease activity. Synovial fluid from two patients was collected on an anonymous basis as rest material from arthrocentesis at the department of Rheumatology at Groene Hart Ziekenhuis, Gouda, The Netherlands. Because of the anonymous sample collection, no clinical characteristics were available from these donors except for the diagnosis of established, ACPA-positive RA. Eow: every other week.

RA patients who visited the outpatient clinic of the department of Rheumatology at Leiden University Medical Center (LUMC, Leiden, The Netherlands), and who fulfilled the 1987 criteria for RA (see Table I for patient characteristics). Neutrophils were isolated from peripheral blood of five healthy donors. The ethical review board of LUMC approved the study, and patients and healthy donors gave written informed consent for participation. Additional SF samples were anonymous collected as rest material from arthrocentesis at the department of Rheumatology at Groene Hart Ziekenhuis, Gouda, The Netherlands.

*Cells*

Stably transfected FLAG-tagged human FcγR Chinese Hamster Ovary (CHO) cells were kindly provided by M. Daëron (Institut Pasteur, Paris, France) (8). Wild-type CHO cells were cultured in Roswell Park Memorial Institute (RPMI) medium (Gibco, Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin (100U/ml). Transfected CHO cells expressing hFcγRIIA (genotype H131 and R131) and hFcγRIIB were cultured in RPMI with 10% FCS, penicillin/streptomycin (100U/ml), 1% non-essential amino acids (Sigma-Aldrich, St. Louis, USA) and 1% geneticin (Gibco). hFcγRI and hFcγRIIIA (genotype F176 and V176) transfected CHO cells were cultured with additional 0.25% zeocin (Invivogen, San Diego, USA).

*Neutrophil isolation*

Blood was collected in anticoagulant EDTA tubes and diluted with PBS before Ficoll-Paque gradient centrifugation (LUMC pharmacy). Bottom fraction containing erythrocytes was lysed by hypotonic shock with cold water for 20 seconds before stopping the lysis with 10x PBS pH7.4 without calcium and magnesium (Gibco). Purity of neutrophil preparations was >95% as determined by CD15 and CD16 expression using flow cytometry. Neutrophils were cultured in 96-wells flat bottom plates at a density of $4x10^6$ cells/ml in serum-free X-VIVO 15 medium without gentamicin and phenol red (Lonza, Verviers, Belgium) and 1% penicillin/streptomycin (100U/ml), to limit monomeric IgG binding to FcγRI. Neutrophils were either stimulated with 100 ng/ml recombinant human interferon gamma (IFNγ) (Peprotech, Rocky Hill, USA) or left unstimulated. After overnight incubation at 37°C and 5% $CO_2$, neutrophils were immediately used for IC binding assays.

*Antibodies*

Individual FcγR expression on CHO cell lines was confirmed with antibodies against hCD64-PE (clone 10.1), panhCD32-PE (clone FLI8.26) and hCD16-PE (clone 3G8, all BD Biosciences, San Diego, USA), hCD32B-Alexa Fluor 488 (clone 2B6, MacroGenics, Rockville, USA) and hCD32A-

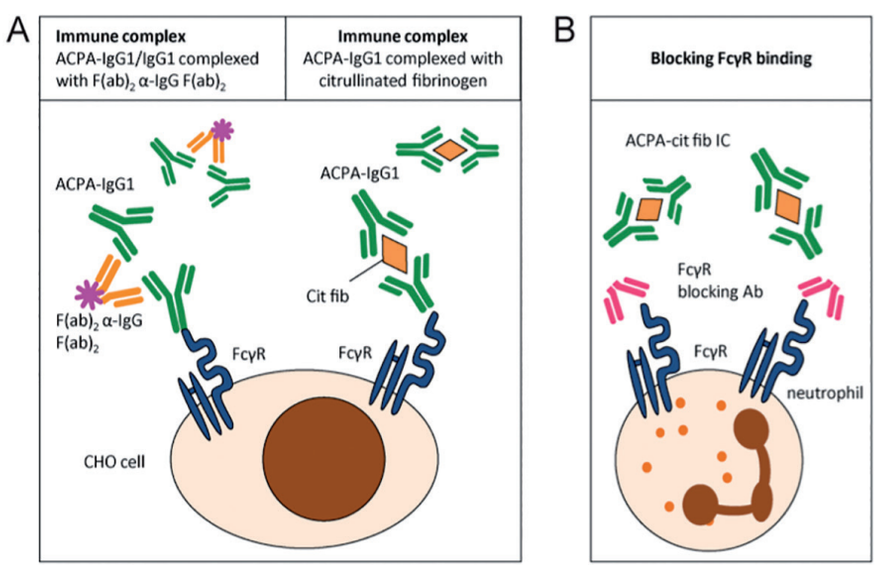


**Fig. 1.** Schematic representation of the immune complex assay.
**A**: Immune complexes were formed using isolated ACPA-IgG1 or control IgG1 and a labelled $F(ab)_2$ α-IgG $F(ab)_2$ antibody, or by incubating isolated ACPA-IgG1 together with citrullinated fibrinogen. These immune complexes were added to FcγR expressing CHO cells to determine (ACPA-)IgG1 IC binding to the individual FcγR.
**B**: To confirm ACPA-cit fib IC binding to neutrophils, neutrophils were pre-incubated with $F(ab)_2$ anti-FcγR blocking antibodies before adding ACPA-cit fib IC.

FITC (clone IV.3, Stemcell Technologies, Vancouver, Canada). Anti-FLAG M2-FITC antibody (Sigma-Aldrich) was used to determine cellular FcγR expression levels. To characterise neutrophils, we used anti-hCD15-APC (clone HI98) and anti-hCD16-FITC (clone 3G8, both BD Biosciences).

*Isolation of ACPA-IgG1 and control IgG1 from plasma and SF*

Total ACPA-IgG and control IgG (IgG depleted of ACPA) were isolated from plasma and SF by fast protein liquid chromatography (ÄKTA, GE Healthcare, Uppsala, Sweden) as described previously (19). In short, HiTrap Streptavidin HP 1 ml columns (GE Healthcare) were coupled with biotinylated cyclic citrullinated peptide 2 (CCP2) or control (arginine) peptide prior to sample loading onto the column. While control IgG (non-specific) antibodies were recovered in flow-through fractions, ACPA-IgG (specific) antibodies were bound to the CCP2 column and as such, were eluted with 0.1M glycin HCl, pH 2.5 and directly neutralised with 2M Tris. The CCP2 peptide used for isolation detects the vast majority of citrulline-specific antibody reactivities, and ELISA analysis confirmed that there was no remaining CCP2 reactivity in the control IgG1 fraction after ACPA isolation (data not shown). ACPA-IgG and control IgG were further purified on HiTrap protein G and protein A 5 ml columns (GE Healthcare) to obtain IgG1, 2 and 4. Analysis of the fractions by ELISA gave no indication for the co-purification of IgM-rheumatoid factor (RF, data not shown), and size determination of monomeric and complexed ACPA-IgG and control IgG by asymmetrical flow field-flow fractionation (AF4) did not suggest the presence of IgG-RF in the ACPA preparations (Supplementary Fig. 3).

As IgG1 is the most abundant IgG subclass of ACPA, and to control for variations in FcγR binding due to differences in IgG subclass composition in our samples, we focussed our study on ACPA-IgG1 using IgG1 depleted of ACPA-IgG1 (termed "control IgG1") as control (8, 20). To obtain ACPA-IgG1 and control IgG1, fractions were further purified with a HiTrap Streptavidin HP 1 ml column (GE Healthcare) coupled with biotinylated monoclonal anti-human IgG1 (Sigma-Aldrich). IgG1 purification was confirmed by PeliClass human IgG subclass ELISA kit (Sanquin, Amsterdam, The Netherlands) (Supplementary Fig. 1). After isolation, samples were concentrated by centrifugal ultrafiltration (Amicon Ultra-15, 50K MWCO, Merck Millipore, Tullagreen, Ireland) and desalted using Zeba desalt spin columns (7K MWCO, Thermo Scientific, Rockford, USA) according to manufacturer's instructions. Concentrations of ACPA-IgG and control IgG were measured at 280 nm with a NanoDrop spectrophotometer, whereas ACPA-IgG1 and control IgG1 were measured with Pierce BCA Protein Assay (Thermo Scientific), as these samples contained lower protein concentrations. From one patient, ACPA-IgG1 and IgG1 concentrations were only enough to make IC with citrullinated fibrinogen.

*Fc glycosylation analysis*

Fc-linked glycosylation profiles of ACPA-IgG1 and control IgG1 was analysed by LC-MS, as previously described (21, 22). Briefly, antibodies were dried in a vacuum centrifuge and digested with 200 ng trypsin in 40 μL ammonium bicarbonate buffer during overnight incubation at 37°C. The resulting (ACPA)-IgG1 glycopeptides were separated and analysed on an Ultimate 3000 UPLC system (Dionex Corporation, Sunnyvale, USA) coupled to a maXis™ Impact Ultra-High Resolution Q-TOF mass spectrometer (MS) (Bruker Daltonics, Bremen, Germany) (21, 22). Quality of mass spectra was evaluated based on intensities of total (ACPA)-IgG1 glycoforms. Data processing and calculations of the level of galactosylation, sialylation, and fucosylation residues of (ACPA)-IgG1 were performed as described (21, 22).

*Immune complex binding assay*

Isolated ACPA-IgG1 or control IgG1 (0.3 μg/ml) were incubated with 0.5 μg/ml Alexa Fluor 647 labelled polyclonal $F(ab')_2$ anti-human IgG antibody specific for IgG, $F(ab')_2$ fragment (Jackson ImmunoResearch, Baltimore Pike, USA) for 30 minutes at 37°C (Fig. 1A). For binding studies of IC containing a natural antigen, fibrinogen (Sigma-Aldrich) was biotinylated using the ImmunoProbe Biotinylation Kit (Sigma-Aldrich). Biotinylated fibrinogen was either citrullinated for 3 hours at 37°C as described (23) or left uncitrullinated by omitting $CaCl_2$ from the reaction. ACPA-IgG1 or total ACPA-IgG (5 μg/ml) were incubated with 0.5 μg/ml biotinylated (citrullinated) fibrinogen for 30 minutes at 37°C. We estimated the size of ACPA-IgG1 and control IgG1 IC by asymmetrical flow field-flow fractionation and observed that both IC preparations were of comparable sizes and contained similar proportions of monomeric and complexed IgG1 (Supplementary methods and Supplementary Fig. 3).

After the formation of IC, 25 μl of IC were added to $1x10^5$ FcγR-transfected CHO cells for 1 hour at 4°C (Fig. 1A). To allow detection of binding differences, the IC concentration was chosen below saturation levels based on titration curves for each CHO cell line, which was in line with previously reported concentrations (supplementary Fig. 2) (8). Subsequently, flow cytometric analysis was performed on the LSRFortessa (BD Biosciences); data were analysed using FlowJo v. 7 (FlowJo, Ashland, USA).

For neutrophil experiments, $2x10^5$ neutrophils were pre-incubated for 45 min at 4°C with 80 μg/ml anti-CD32 (clone 7.3) or anti-CD64 (clone 10.1) $F(ab')_2$ blocking antibodies (Ancell, Bayport, USA) before adding total ACPA-IgG complexed with citrullinated fibrinogen for 1 hour at 4°C (Fig. 1B). Next, neutrophils were stained for 30 min at 4°C with streptavidin-PE (eBioscience, San Diego, USA) for the detection of biotinylated ACPA-cit fib IC. Flow cytometric analysis of neutrophils was performed on the LSRII (BD Biosciences).

*Statistical analysis*

Data were analysed using GraphPad Prism v. 6.05 (La Jolla, USA). Comparison of ACPA-IgG1 and control IgG1 was assessed as a non-parametric Wilcoxon matched-pairs signed rank test.

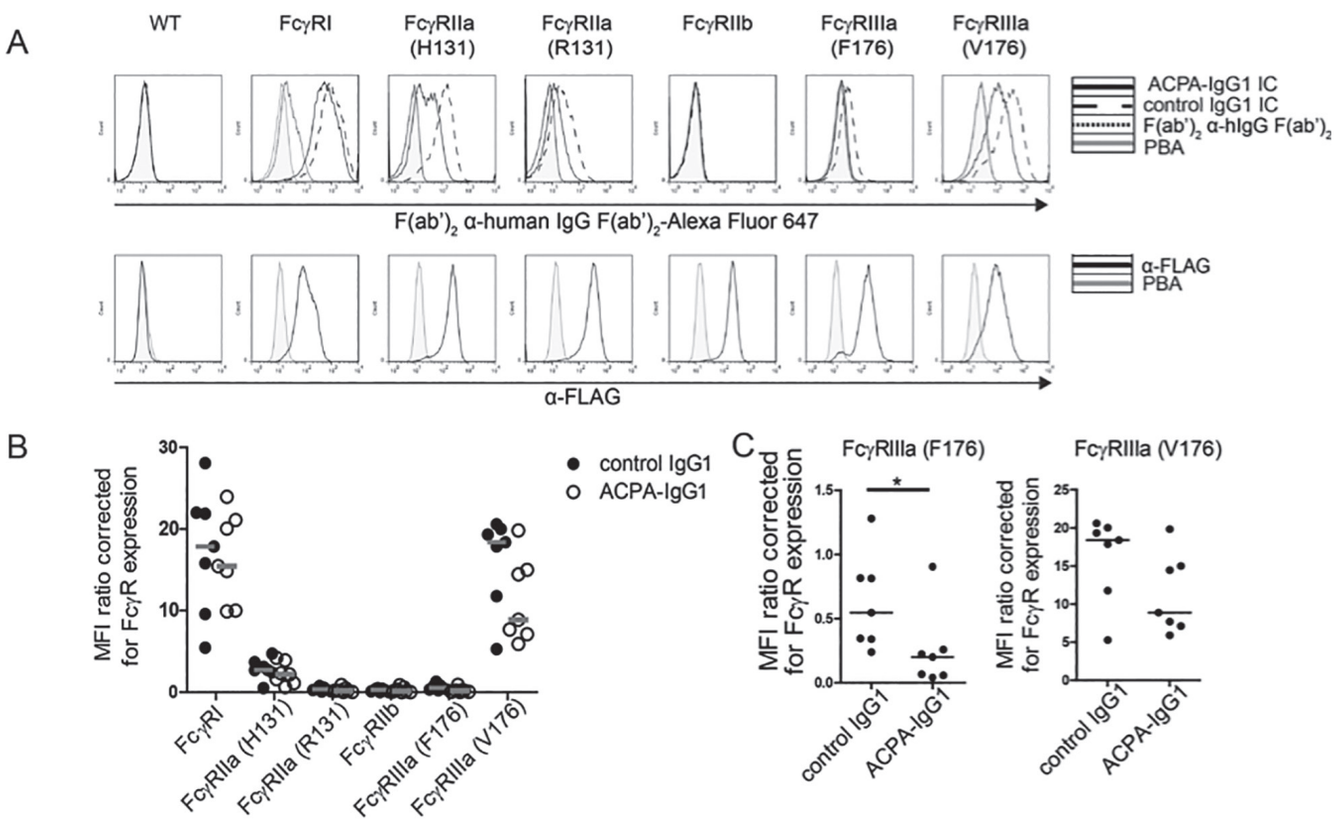


**Fig. 2.** ACPA-IgG1 and control IgG1 IC bind activating FcγRI and FcγRIIIA on transfected CHO cell lines. **A**: Representative plots of ACPA-IgG1 and control IgG1-IC binding to FcγR-transfected CHO cell lines from one RA patient. WT; wild type, PBA; PBS/BSA solution in which antibodies are diluted. **B**, **C**: Binding of ACPA-IgG1 and control IgG1 IC of 7 RA patient samples to FcγR-transfected CHO cell lines displayed by the Mean Fluorescence Index (MFI) ratio corrected for the FcγR expression. Each dot represents control IgG1 IC (black dot) or ACPA-IgG1 IC (white dot) formed with isolated (ACPA-) IgG1 from one RA patient. The bar indicates the median of the results. Statistical analysis was performed using the non-parametric Wilcoxon matched-pairs signed rank test.* represents a *p*-value of <0.05.

Inhibition of ACPA-cit fib IC binding to FcγR on (un)stimulated neutrophils was assessed using the Kruskal-Wallis test followed by a Dunn's multiple comparisons test.

## Results

### *ACPA-IgG1 and control IgG1 immune complexes predominantly bind to activating FcγR*

To study the binding of ACPA-IgG1 IC to individual FcγR, we used an *in vitro* system of CHO cell lines transfected with individual FLAG-tagged human FcγR. ACPA-IgG1 and control IgG1 IC were incubated with FcγR-transfected CHO cells to determine specific binding profiles of these IC to individual FcγR (Fig. 1A, 2A). We corrected for differences in FcγR expression by calculating the ratio of the Mean Fluorescence Index (MFI) of IC binding divided by the MFI of the FcγR-FLAG expression. Interestingly, ACPA-IgG1 IC mainly bound to activating receptors FcγRI and FcγRIIIA (V176), whereas much lower binding was observed to FcγRIIA (H131), the inhibiting receptor FcγRIIB and activating FcγR with less frequent polymorphisms (Fig. 2B). ACPA-IgG1 IC had similar binding capacities as control IgG1IC to FcγRI and FcγRII, but showed lower binding to FcγRIIIA (F176) and (V176) (Fig. 2C).

To mimic more closely the natural situation of ACPA-IC in the context of RA pathogenesis, we also generated IC by incubating ACPA-IgG1 with biotin-labelled citrullinated fibrinogen (cit fib), a natural antigen of ACPA. A similar binding pattern to the different FcγR was observed for ACPA-cit fib IC where, again, strongest binding was detected to FcγRI and FcγRIIIA (V176) (Fig. 3A-B).

### *ACPA-IgG1 Fc glycosylation profiles*

The absence of core-fucosylation in the Fc-linked *N*-glycan can enhance binding of antibodies to FcγRIIIA (13, 24). To examine if differences in Fc-linked glycan profiles of ACPA-IgG1 and control IgG1 could account for the differential FcγRIII binding profiles observed in Figure 2C, we determined the Fc glycosylation profiles of our samples. No differences were observed for galactose, bisecting GlcNAc and sialic acid residues (Fig. 4). However, we did observe a significant increase in ACPA-IgG1 Fc core fucosylation, in line with previous reports (22, 25). Together, these results indicate that ACPA-IgG1 core-fucosylation is likely responsible for reduced binding of ACPA-IgG1 IC to FcγRIIIA.

### De novo *surface expression of FcγRI by activated neutrophils is paralleled by enhanced binding of ACPA-IC*

We were intrigued by the strong binding capacity of ACPA-IgG1 IC to FcγRI on transfected CHO cell lines, given the reported expression of this

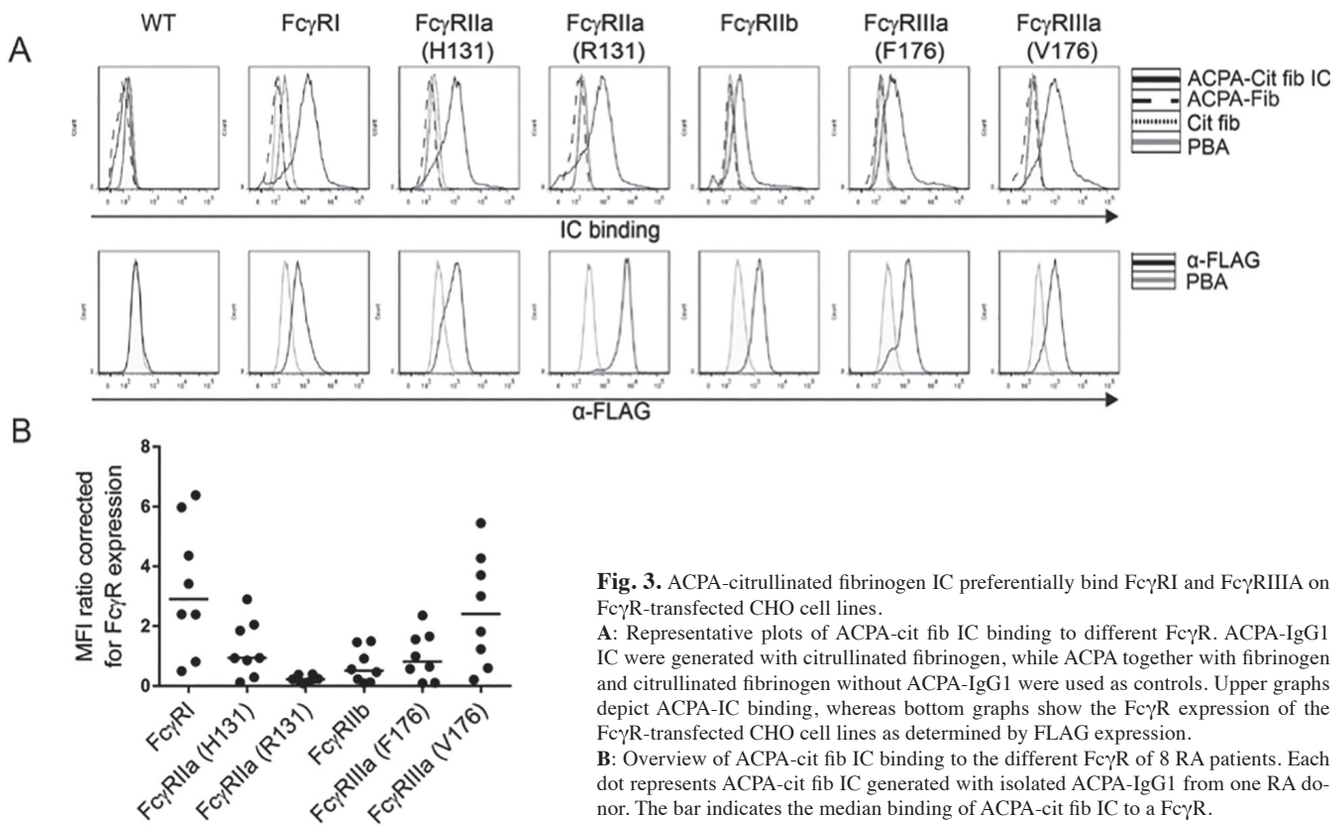


**Fig. 3.** ACPA-citrullinated fibrinogen IC preferentially bind FcγRI and FcγRIIIA on FcγR-transfected CHO cell lines.
**A**: Representative plots of ACPA-cit fib IC binding to different FcγR. ACPA-IgG1 IC were generated with citrullinated fibrinogen, while ACPA together with fibrinogen and citrullinated fibrinogen without ACPA-IgG1 were used as controls. Upper graphs depict ACPA-IC binding, whereas bottom graphs show the FcγR expression of the FcγR-transfected CHO cell lines as determined by FLAG expression.
**B**: Overview of ACPA-cit fib IC binding to the different FcγR of 8 RA patients. Each dot represents ACPA-cit fib IC generated with isolated ACPA-IgG1 from one RA donor. The bar indicates the median binding of ACPA-cit fib IC to a FcγR.

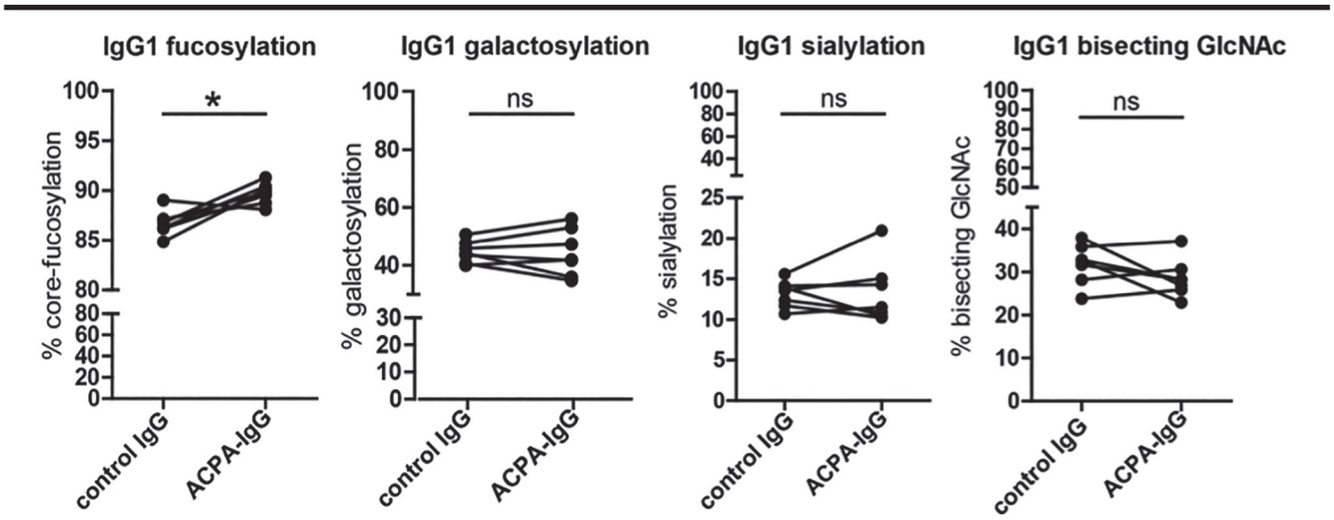


**Fig. 4.** Fc glycosylation profile of ACPA-IgG1 and control IgG1. IgG1 Fc core-fucosylation, galactosylation, sialylation and bisecting GlcNAc was measured from 7 ACPA-IgG and control IgG samples by liquid chromatography mass spectrometry. Statistical analysis was performed with a Wilcoxon matched-pairs signed rank test. *represents a *p*-value of <0.05.

receptor by activated neutrophils in the synovial compartment. As neutrophils are the most prominent cells present in SF, and as ACPA-IC are likely to be present in this compartment, we used neutrophils as a model to investigate the binding of ACPA-cit fib IC to FcγRI. While FcγRI is constitutively expressed by some immune cells, neutrophils only express this receptor upon activation (26). Thus, neutrophils were activated or left in a resting state, followed by incubation with ACPA-cit fib IC to assess FcγR binding by flow cytometry (Fig. 5A). Upon overnight incubation, we consistently observed two $CD15^{+}CD16^{+}$ neutrophil populations. We specifically gated the $CD15^{high}CD16^{high}$ population to exclude (pre-)apoptotic neutrophils (Supplementary Fig. 4). Of note, stimulation with IFNγ not only induced FcγRI expression but also induced a non-significant increase in binding of ACPA-cit fib IC to neutrophils. Importantly, FcγRII and FcγRIII expression remained unchanged (Fig. 5B-C). Together, these results indicate that ACPA-cit fib IC could bind FcγRI on activated neutrophils.

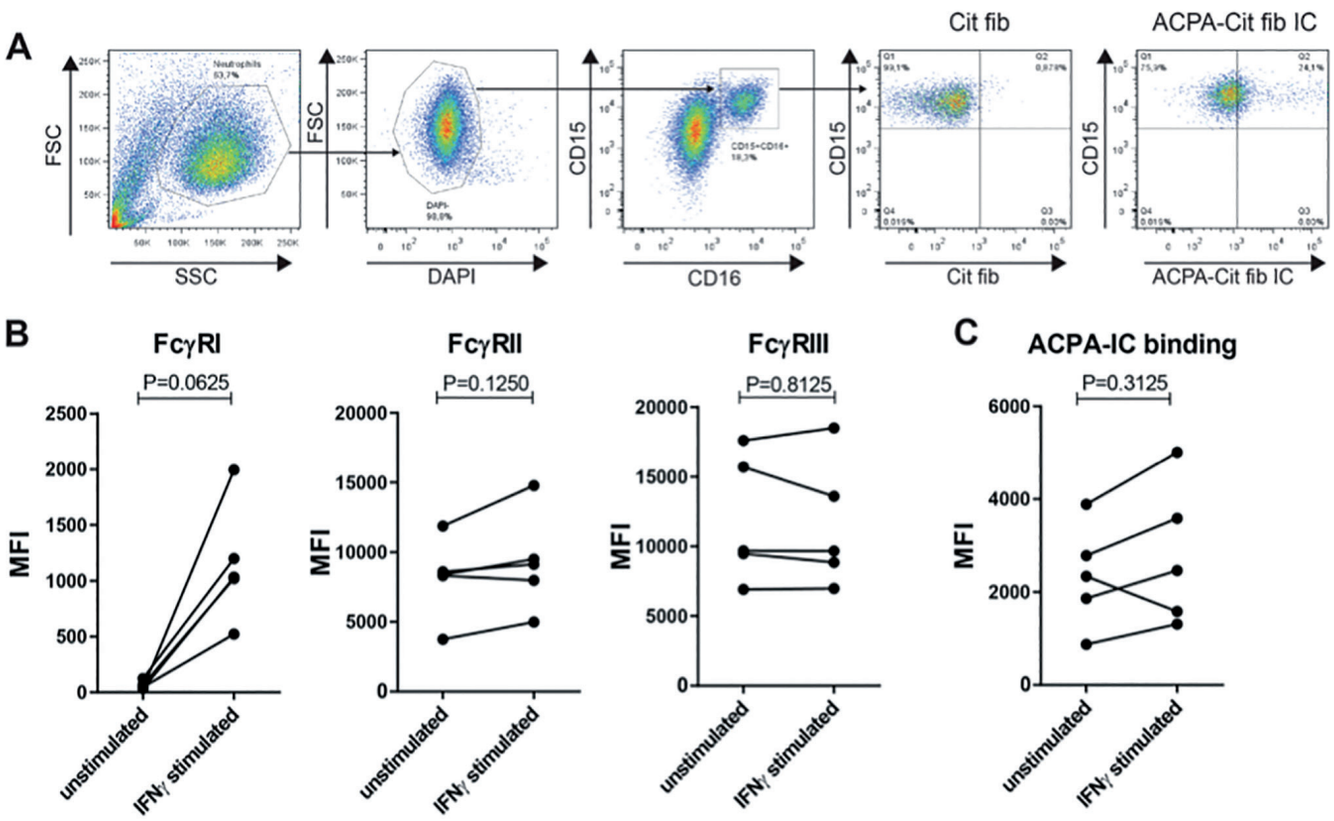


**Fig. 5.** Enhanced binding of ACPA-citrullinated fibrinogen IC to neutrophils is paralleled by increased FcγRI expression. Neutrophils isolated from 5 healthy donors were cultured overnight with or without IFNγ in serum-free medium. Neutrophils were then incubated with total ACPA-IgG cit-fib IC and binding was measured with flow cytometry.
**A**: Representative plots and gating strategy. FSC; forward scatter, SSC; side scatter; DAPI; nucleic acid staining.
**B**: FcγRI, FcγRII and FcγRIII expression on unstimulated and IFNγ-stimulated neutrophils from 5 healthy donors was measured using the Mean Fluorescence Index (MFI). Lines connect unstimulated and IFNγ-stimulated neutrophils from the same healthy donor.
**C**: MFI of ACPA-IgG cit-fib IC binding to unstimulated and IFNγ-stimulated neutrophils from 5 healthy donors is depicted. A non-parametric Wilcoxon test for matched pairs was used to evaluate statistical differences.

*ACPA-IC binding to neutrophils is dependent on FcγRI and FcγRIIA*

To confirm that ACPA-cit fib IC binding to activated neutrophils is, at least in part, mediated by FcγRI, we pre-incubated neutrophils with F(ab')$_2$ fragments of blocking antibodies against FcγRI or FcγRII or with a combination of these prior to ACPA-cit fib IC incubation (Fig. 1B). ACPA-cit fib IC binding to unstimulated neutrophils, *i.e.* in the absence of FcγRI expression, could be inhibited by 74% upon blocking FcγRIIA (confidence interval (CI) 1043–4122 without and CI 325–988 with FcγRII blocking antibodies) (Figure 6A-C), while, as expected no inhibitory effect of FcγRI blockade was observed. Interestingly, however, ACPA-cit fib IC binding to IFNγ-activated neutrophils could be inhibited by 29% upon blocking FcγRI (CI 1502–5276 without and CI 979–3767 with FcγRI blocking antibodies) (Fig. 6A-C). In addition, blocking of both FcγRI and FcγRII on IFNγ-stimulated neutrophils further reduced, although modestly, ACPA-cit fib IC binding (Fig. 6B, 5C). Together, these results indicate that, next to FcγRIIA, ACPA-cit fib IC can bind FcγRI on activated neutrophils.

To summarise, ACPA-IgG1 IC binding to FcγR transfected CHO cells mainly identified ACPA-IgG1 IC binding to FcγRI and FcγRIIIA. These results could be verified using IC with ACPA-IgG1 and citrullinated fibrinogen, which more closely reflect natural occurring IC in RA. Furthermore, the difference in ACPA-IgG1 IC and IgG1 IC binding to FcγRIIIA might be explained by the difference in Fc fucosylation. To translate our findings in a more representative setting, we studied the binding of ACPA-cit fib IC to healthy neutrophils, which expressed all three FcγR upon stimulation with IFNγ. Blocking FcγRI and FcγRII revealed that ACPA-cit fib IC can also bind FcγRI, in addition to FcγRII.

## Discussion

RA-specific autoantibodies, ACPA, are thought to contribute to disease pathogenesis as the presence of these autoantibodies strongly correlates with progressive and severe disease (1, 2). Here, we studied binding of ACPA-IgG IC to individual FcγR in a stable and standardised *in vitro* system using single FcγR-transfected CHO cell lines. This approach is unique, as it controls for the complexity of FcγR expression levels on cell surfaces. Thereby, it can identify ACPA-IgG IC binding characteristics that might remain unnoticed if primary immune cells are studied using read-out systems such as cytokine secretion. In this experimental setting, we

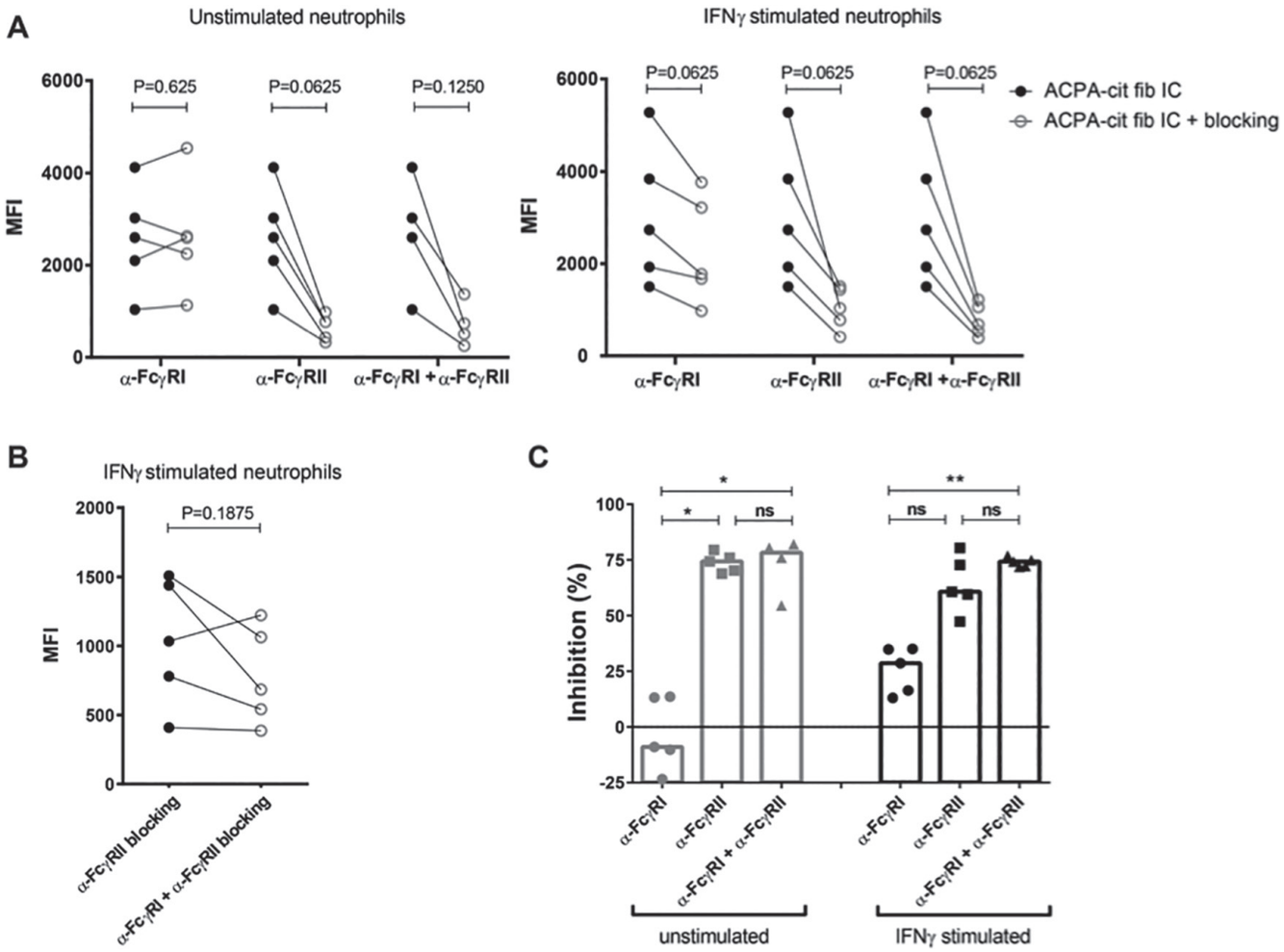


**Fig. 6.** Blocking FcγRI and FcγRII reduces ACPA-citrullinated fibrinogen IC binding to neutrophils. **A**: ACPA-cit fib IC binding to unstimulated or IFNγ-stimulated neutrophils with (white dots) or without (black dots) pre-incubation of FcγRI and FcγRII or **B**: both blocking antibodies. **C**: Percentage of inhibition of ACPA-cit fib IC binding under FcγR blocking conditions compared to non-blocking conditions. Bar graphs represent the median inhibition and dots indicate the individual inhibition of ACPA-cit fib IC binding to healthy donor neutrophils (n=5). Statistics were performed with a Wilcoxon matched-pairs signed rank test and Dunn's multiple comparisons test. * and ** represent a *p*-value of <0.05 and <0.01 respectively.

observed that ACPA-IgG1 IC strongly bind to FcγRI. Using ACPA-IgG complexed with citrullinated fibrinogen, we sought to further understand whether also human primary immune cells that express various FcγR simultaneously, would show binding of ACPA-IgG IC to FcγRI. Indeed, we observed that IFNγ-stimulated human neutrophils, which *de novo* express FcγRI, bind ACPA-cit fib IC via this receptor. This could be especially relevant in the context of synovial inflammation as neutrophils isolated from SF of RA patients exhibit an activated state and express FcγRI (27-30).

FcγRI is unique in its high affinity for monomeric IgG present in serum. Therefore, FcγRI expressed by immune cells is constantly occupied by serum IgG to facilitate sampling of extracellular antigens (6, 31). IC, however, can compete with monomeric IgG for *de novo* expressed FcγRI molecules (5, 31, 32). Interestingly, neutrophils constitutively express FcγRII and FcγRIII, while the expression of FcγRI requires induction by, for example, IFNγ. As neutrophils represent the majority of immune cells in the SF and as IFNγ is found in this compartment, it is conceivable that FcγRI-triggering of neutrophils is of importance in RA.

Previous studies that investigated the inflammatory potential of ACPA-containing IC mainly focussed on a role for FcγRIIA expressed by macrophages in the pathogenesis of RA. Immobilised (insoluble) ACPA-containing IC were shown to induce TNF-α production by macrophages mainly via FcγRIIA, while no significant contribution of FcγRI or FcγRIIIA could be observed (14-16, 33, 34). In our study, we could confirm binding of ACPA-IgG IC to FcγRIIA on both FcγR-transfected CHO cell lines and on human neutrophils, while no binding of ACPA-IgG1 IC to FcγRIIB was observed. However, if comparable numbers of receptors are present on the cell surface, we observed that binding of IC to FcγRI is much more pronounced than to FcγRIIA (Fig. 2B). The difference between our results and previously reported findings might be due to differences in the distribution/composition of FcγR expressed by the cell-types studied, the detection system used (bindings studies *vs*. cytokine secre-

tion), and differences in FcγR engagement between soluble and insoluble IC (35). Here, we observed that blocking FcγRI on activated neutrophils reduces ACPA-IgG IC binding by approximately 30%. This blocking effect is likely an underestimation, as the FcγRI blocking antibody used blocked only 56% of the specific ACPA-cit fib IC binding to FcγRI on CHO cells, despite optimised concentrations and its specificity for this receptor (Supplementary Fig. 5). In contrast, anti-FcγRII F(ab')$_2$ fragments inhibited ACPA-cit fib IC binding to CHO cells by 82%, demonstrating its higher inhibitory potential. In addition, previous data indicate that more FcγRII molecules are present on the neutrophil cell surface compared to the levels of FcγRI (36). This might explain why, despite strong ACPA-IC binding to FcγRI expressed by CHO cells, the strongest reduction of ACPA-IC binding on neutrophils was still observed upon blocking FcγRII.

We did not investigate the binding of ACPA-cit fib IC to FcγRIII on neutrophils as FcγRIII was used as a neutrophil cell-surface marker in our analyses. However, based on our ACPA-IC binding data using transfected CHO cells, it is likely that ACPA-IC also bind FcγRIII on neutrophils. Compared to control IgG IC, however, the high degree of ACPA-IgG Fc core-fucosylation reduces the affinity of ACPA-IgG IC for this receptor (Fig. 2C). This might suggest that FcγRIIIA-mediated effector mechanisms such as antibody-dependent cell-mediated cytotoxicity contribute less prominently to ACPA pathogenicity (24).

Finally, we noted donor dependent variations in ACPA-IgG IC binding to various FcγR (Fig. 2B), despite our focus on the IgG1 subclass of ACPA and control IgG. ELISA analysis excluded the presence of IgM-RF in our ACPA preparations and size fractionation measurements of both ACPA-IgG and control IgG IC were comparable, thereby indicating that no IgG-RF was co-purified. The latter, however, cannot be fully excluded due to inherent difficulties in determining the presence of IgG-RF. We further assessed the composition of Fc-linked glycans in both preparations. Except for differences in fucosylation between ACPA-IgG1 and control IgG1 that are known to modulate IC binding to FcγRIIIA, no significant differences were noted with regard to the other glycoforms. However, due to the relatively low number of samples analysed and the diversity of Fc-linked *N*-glycans, it is still possible that variance in Fc-glycosylation accounts, at least in part, for the donor variations observed. Finally, with regard to ACPA-IgG IC generated with citrullinated fibrinogen (Fig. 3), the polyclonality of ACPA and, thus, the affinity of different ACPA-IgG molecules for citrullinated fibrinogen could have contributed to the observed variations between donors. Together, these considerations reflect the complexity of ACPA and of the FcγR system and thus, the importance of tedious controls in the assessment of contributions of individual FcγR to disease processes, as performed here.

In conclusion, we here dissected binding characteristics of ACPA-IgG IC to individual FcγR and report a particular role for ACPA-cit fib IC binding to FcγRI on activated neutrophils. Next to FcγRIIA-mediated effects described previously, these observations provide additional arguments for the pathogenic role of ACPA in RA, especially in the synovial compartment in which neutrophils exhibit an activated state, express FcγRI and where ACPA-IgG IC are abundant. Therefore, detailed analysis of the Fc-mediated downstream effector mechanisms of ACPA-IgG IC binding to FcγRI is warranted.

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