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

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# CHAPTER 5

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## **Rheumatoid Factors Do Not Preferentially Bind to ACPA-IgG or IgG with Altered Galactosylation**

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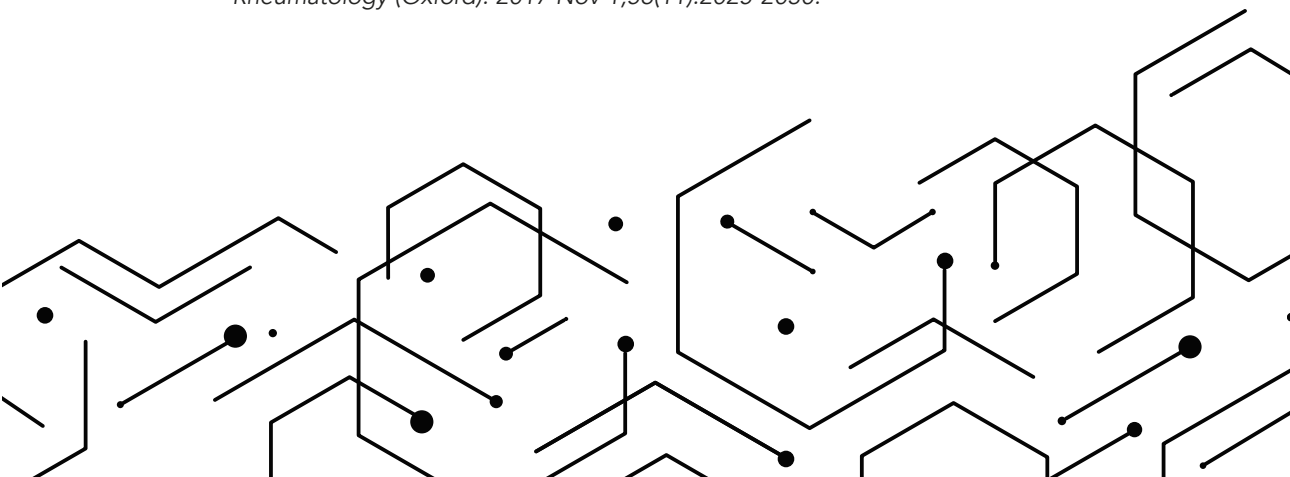
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## ABSTRACT

**Objectives:** Recent reports on interactions between the two most prominent RA-related autoantibodies, rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPAs), led us to investigate whether RFs preferentially interact with ACPA-IgG over non-ACPA IgG. Additionally, interactions of RFs with IgG with altered galactose content in the Fc-domain were examined, since ACPA-IgGs have been shown to have decreased Fc-galactose content in RF positive patients.

**Methods:** (Auto)antibody interactions were studied in a Surface Plasmon Resonance imaging (SPRI) assay and with ELISA. Target antibodies were isolated from RA patient plasma (polyclonal ACPA- and non-ACPA-IgG) or recombinantly produced to obtain monoclonal IgG with well-defined Fc galactose content. Interacting autoantibodies were studied using autoantibody positive patient sera and two recombinantly produced IgM-RFs.

**Results:** The 41 RF-positive RA patient sera studied showed similar RF binding to ACPA- and non-ACPA-IgG and no differences in binding to IgG with normal, high or low levels of Fc-galactosylation. Two monoclonal IgM-RFs, one interacting with the CH2-CH3 interface and one binding close to the C-terminal end of the CH3 domain showed no influence of the Fc-glycan on IgG binding by IgM-RF.

**Conclusion:** Although interactions between RF and ACPA may play a role in inflammatory processes in RA, RFs do not preferentially interact with ACPA-IgG over non-ACPA-IgG nor with agalatosylated IgG over IgG with normal or high galactosylation.

## INTRODUCTION

Rheumatoid factors (RFs) are autoantibodies, primarily of the IgM isotype, that bind to the constant (Fc-) domain of IgG. RFs were the first autoantibodies discovered to be associated with rheumatoid arthritis (RA) [1], are part of the RA classification criteria [2] and have prognostic value [3]. Anti-citrullinated protein antibodies (ACPAs) recognize arginine amino acid residues converted enzymatically into citrulline and were linked to RA more recently [4]. Both RFs and ACPAs have been implicated in the pathogenesis of RA. Recently there has been an increased interest in the effects of RF-ACPA interactions [5, 6], since RF could bind IgG-ACPAs and RF and ACPA are often found together in RA [7]. Likewise, several studies suggest an interaction between ACPA and RF as their combined presence is found in patients, but their presence is discordant in their (seropositive) healthy relatives [8]. The combined presence is associated with enhanced bone marrow edema as well as with higher levels of pro-inflammatory cytokines and increased acute phase proteins and disease activity [7, 9]. In vitro it was shown that RF can enhance monocyte activation by ACPA-containing immune complexes [7] and unpublished data suggest that crosslinking of ACPA-Fc's by RF may enhance binding of ACPAs to their citrullinated targets by creating immune complexes with higher avidity. However, it is unknown whether RFs preferentially bind ACPA-IgG over non-ACPA-IgG. We hypothesized that RFs might preferentially bind ACPAs over non-ACPA-IgGs based on results showing that ACPA-IgGs in RF-positive patients have a lower galactose content of the glycans in the IgG Fc-domain compared to ACPAs in RF-negative patients [10]. This is relevant in the light of other studies showing better binding of RF to IgG with lower Fc-galactosylation [11, 12]. Here, we used enzyme-linked immunosorbent assays (ELISAs) and a Surface Plasmon Resonance imaging (SPRi) array to compare binding of RF to ACPA-IgG and non-ACPA-IgG isolated from plasma of RA patients. Furthermore, we investigated whether the binding of RFs is influenced by the degree of galactosylation of the IgG-Fc domain by evaluating binding of serum RF and recombinant monoclonal RFs to recombinant monoclonal IgG with different galactosylation levels.

## METHODS

### Isolation of ACPA-IgG and non-ACPA-IgG from plasma

ACPA-IgG and non-ACPA-IgG were isolated from plasma of three RA patients by affinity chromatography, as previously reported [13]. The ACPA-IgG fractions showed high anti-citrulline reactivity in ELISA, whereas the non-ACPA-IgG fractions showed anti-citrulline reactivity at background (anti-arginine) levels (Supplementary Table S5.1).

### Production of monoclonal antibodies with different galactosylation levels

Anti-human rhesus D heavy and light chain were sequenced from a single human B cell clone from a hyper immunized donor [14]. A single-gene vector containing anti-D or anti-2,4,6-trinitrophenol (TNP) IgG1 heavy- and kappa-light chain encoding sequences was cloned as described previously [15]. IgGs were produced in HEK-freestyle cells. Glyco-engineering of IgGs and analysis of Fc-glycans (Supplementary table S5.2) by mass spectrometry was performed as previously described. [16].

### Production of monoclonal rheumatoid factors

Two monoclonal IgM-RFs(mRFs) were produced. Variable heavy and variable light chain amino acid sequences for mRFs “RF 61” [17] and “RF-AN” [18] were retrieved from the Protein Data Bank [19], accession codes: 2J6E, 1ADQ.

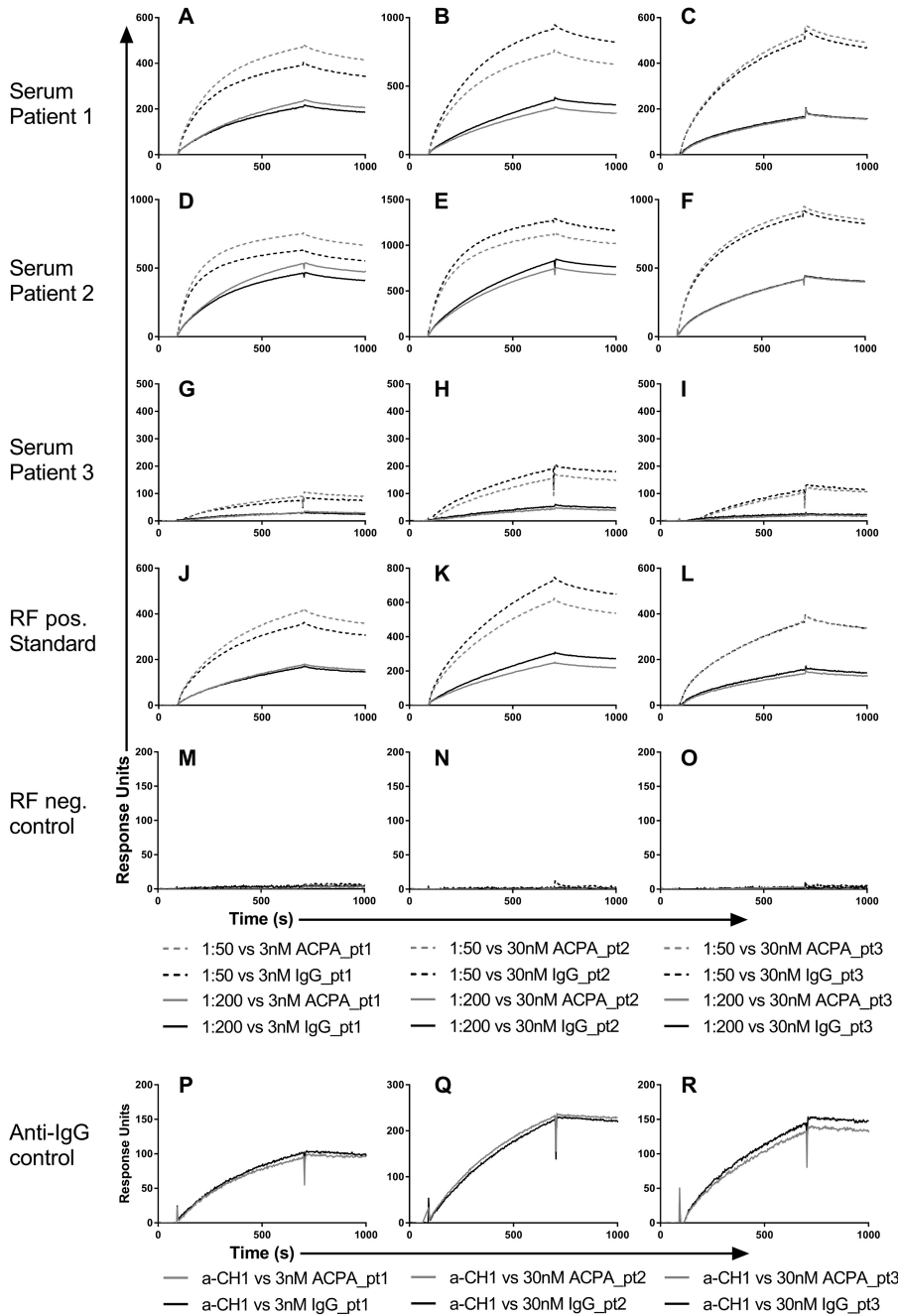
### Surface Plasmon Resonance imaging (SPRi)

For the SPRi experiments the IBIS MX96 imager was used. ACPA-IgG and non-ACPA-IgG isolated from three patients and the three differently galactosylated monoclonal anti-D antibodies were spotted at 30nM, 10nM and 3nM spotting concentrations onto pre-activated Easy2Spot G-type sensors. Samples were flowed over the sensor for 5 or 10 minutes in the association phase, followed by a 5-minute dissociation phase and regeneration of the sensor with a 12-second pulse of 10mM Glycine-HCl.

### Serum samples

Serum from 46 early RA patients from the Amsterdam region was used in the SPRi experiments, 41 RF-positive and 5 RF-negative, determined by commercial assays. All patients signed informed consent forms for use of their samples. The RF-positive standard sample “RELARES” used in the experiments is a national reference serum with a defined IgM-RF level of 200 IU/ml [20].

**Figure 1: Interactions of RF with ACPA-IgG and non-ACPA IgG.** Sensorgrams show interaction of RFs in sera from three different RF-positive RA patients (A-I), RF-positive (J-L) or RF-negative (M-O) reference sera with ACPA-IgG and non-ACPA-IgG isolated from the same three RA patients. The association phase of the curves represents RF in sera (diluted either 1:50 as dotted lines or 1:200 as solid lines) binding under flow to IgG (ACPA grey lines, or non-ACPA black lines) spotted on the SPR-sensor. The dissociation phase starts when the flow of diluted serum is exchanged for buffer and RFs start to dissociate from their targets. Similar coupling of ACPA-IgG and non-ACPA-IgG was confirmed using an anti-CH1 llama antibody fragment flowed over the sensor (P-R). pt = patient.



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## Enzyme linked immunosorbent assays (ELISAs)

For the ELISA assays, target anti-TNP antibodies were diluted to 1  $\mu\text{g/ml}$  and incubated for 2 hours on 96-well flat-bottom plates that had been coated overnight at 4°C with 10  $\mu\text{g/ml}$  TNP-ylated human serum albumin (HSA-TNP) in PBS.

## RESULTS

### Rheumatoid factors do not preferentially bind ACPA-IgG over non-ACPA-IgG

To compare the interactions of serum RFs with ACPA-IgG and non-ACPA-IgG, serum from three RF-positive ACPA-positive RA patients was flowed over a sensor to which ACPA-IgG and non-ACPA-IgG isolated from these same three patients had been coupled. The sensorgrams in Figure 1 show that the RF response in the patient sera, as well as in the RF-positive reference serum, bound the ACPA-IgG and non-ACPA-IgG targets similarly. No binding was observed when RF-negative serum was used. Comparing the two targets, the association- and dissociation phase are similar. The shapes of the binding curves are not different for the RFs in the sera binding to autologous versus allogenic (non-)ACPA-IgG.

To determine whether differential binding of RFs to ACPA-IgG or non-ACPA-IgG could be a feature of a subpopulation of RF-positive RA patients, we next flowed diluted serum from 41 RF-positive and 5 RF-negative RA patients over the sensor. The change in refractive index, expressed as response units (RUs) in the sensorgrams, caused by binding of RF to the IgG-ligands was compared for ACPA- and non-ACPA-IgG at one time point (350 sec.) at the end of the association phase (Figure 2A). Figure 2B shows that for the 38 RF+ sera that gave a sufficient SPR-shift the relative binding to ACPA-IgG versus non-ACPA-IgG is virtually constant, despite an almost 20-fold variation in RF level, suggesting that it is unlikely that RFs preferentially binding to one over the other target make up a significant part of the RF response in sera of RA patients.

### Rheumatoid factors do not preferentially bind to IgG with altered galactosylation

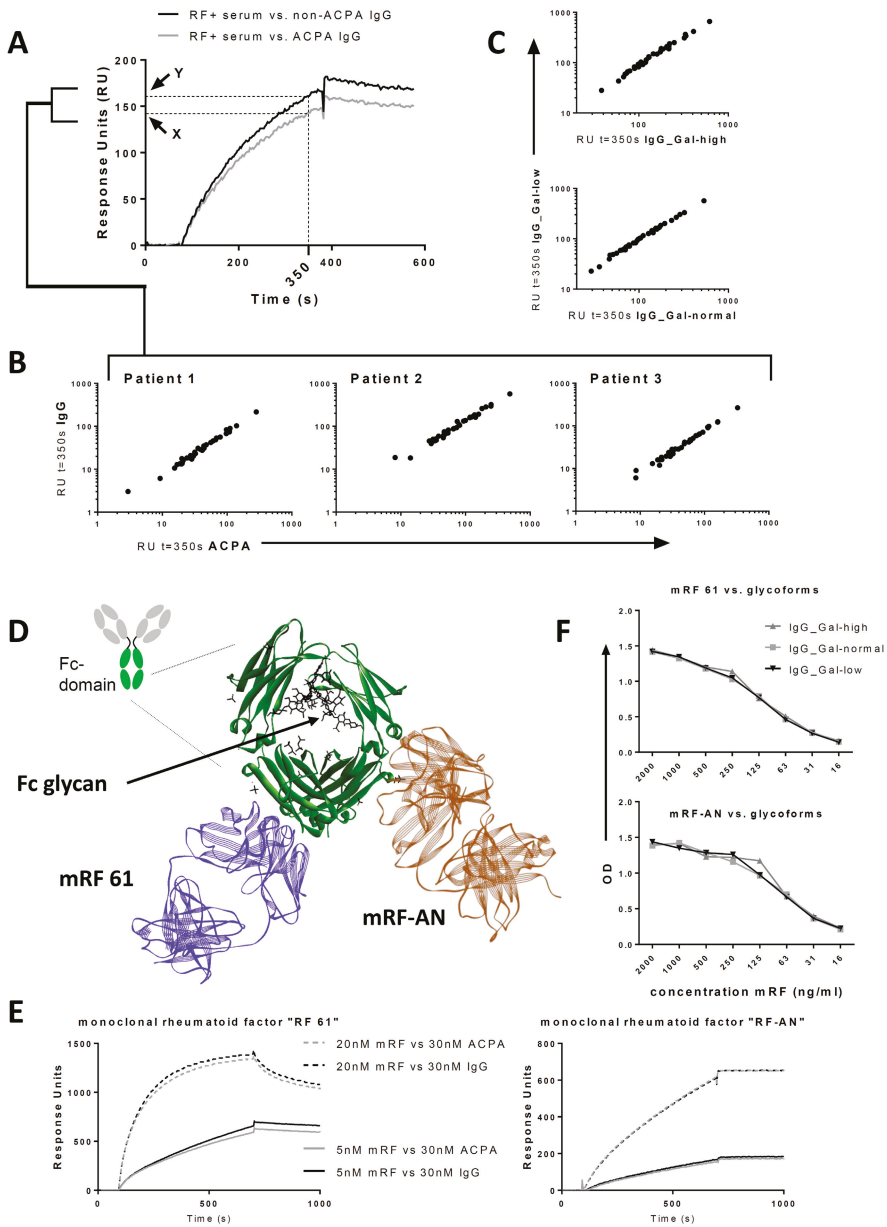
To specifically investigate the influence of the degree of galactosylation of the IgG Fc-glycan in the interaction with serum RFs, we analyzed the binding characteristics of the RF-positive reference serum and the RA patient sera to recombinant monoclonal IgG1 antibodies, glyco-engineered to have different levels of Fc-galactosylation, previously determined by mass spectrometry [16], (Supplementary Table S5.2). Similar to the findings for the ACPA versus non-ACPA comparison, there were no major differences



in RF-association with or RF-dissociation from IgG with normal, low or high galactose content of the Fc-glycan (Supplemental Figure 1A-B). None of the RF responses appeared to have a significant proportion of RFs exclusively binding to one of the three different glycoforms (Figure 2C).

### Interaction of two monoclonal IgM-RFs with ACPAs and differentially galactosylated IgGs

Two monoclonal IgM-RFs were used to investigate whether the specific localization of the RF-IgG interaction site on IgG-Fc influences RF binding of ACPA-IgG and non-ACPA-IgG and differently galactosylated IgGs. Monoclonal RF 61 binds IgG close to the C-terminal end of the CH3 domain [17], at distance from the Fc-glycosylation site, whereas RF-AN binds at the CH2-CH3 interface [18], where the glycan structure might influence the conformation of the RF-epitopes and thereby RF binding (Figure 2D). Both monoclonal RFs bound ACPA- and non-ACPA-IgG similarly (Figure 2E). The observations in Figure 2E that more RF 61 binds in the association phase compared to RF-AN and RF 61 dissociates faster are most likely caused by the higher affinity of RF 61 (KD: ca.  $5 \times 10^{-7}$  M vs ca.  $5 \times 10^{-4}$  M for RF-AN (Supplementary Figure 2)). Due to its higher affinity, RF 61 can probably bind to one or two IgG-Fcs using just one or two Fab-domains, whereas RF-AN would need to make a more polyvalent connection, interacting with more IgG-Fc's resulting in a binding with a higher total *avidity* and slower dissociation. Binding of RF 61 and RF-AN to three IgG-targets with different galactosylation content was evaluated in ELISA. Since coating IgG-targets directly to the plate might induce conformational changes and non-specific binding, we used anti-TNP antibodies specifically binding to TNP-coated plates as targets. Both RF-AN and RF-61 showed equal binding between the three differently galactosylated antibodies at different dilutions of mRF (Figure 2F).



**Figure 2: Binding kinetics of serum RF and two monoclonal RFs to ACPA-IgG, non-ACPA IgG and differently galactosylated IgG.** A) Representative sensorgram of RF positive RA patient serum (1:200 dilution) binding to ACPA- (grey line) and control IgG (black line). The response at 350 seconds was recorded and plotted in B. B) Comparison of RF-responses generated by incubating sera at 1:200 dilution from 38 RF-positive RA patients with an SPR-sensor equipped with control IgG and ACPA-IgG isolated from three different patients and coupled to the SPR-sensor as targets. C) Comparison (as in B) of response units generated by binding of RFs to IgG with low Fc-galactose to IgG with either high (left panel) or normal (right panel) galactosylation of the Fc-domain. D) Interaction sites of mRF 61 and mRF-AN with IgG-Fc. The Fc domain is shown in green, with the N-linked glycan, protruding into the space between the two chains, in black. Fab domains of mRF 61 (purple) and mRF-AN (orange) are shown to illustrate the difference in interaction site with IgG-Fc between the two monoclonal RFs. Figure was created with Discovery Studio 4.5 software using structures 2J6E and 1ADQ from the RSCB Protein Data Bank. E) Sensorgrams showing interaction of mRF 61 (left) and mRF-AN (right) with ACPA-IgG and non-ACPA-IgG isolated from serum of an RA patient. Representative example of three independent experiments with ACPA-IgG and non-ACPA-IgG from three different RA patients. F) Target monoclonal anti-TNP antibodies with low, normal or high galactosylation (1  $\mu\text{g}/\text{ml}$ ) were opsonized onto TNP-ylated HSA-coated plates. Monoclonal mRF 61 and mRF-AN were titrated to compare binding to the anti-TNP antibodies. mRFs did not bind to TNP-ylated HSA.

## DISCUSSION

RF and ACPA are the two major classes of autoantibodies assumed to play a role in RA. Both have predictive value for RA onset and severity. Interestingly, their combined presence is a better marker for severe disease than presence of only RF or only ACPA [7]. While pathogenic properties of RF and ACPA in isolation have been studied in detail, only recently studies have investigated the combination [5-7]. In the present study, we investigated whether RFs would preferentially interact with ACPA-IgG compared to non-ACPA-IgG. This hypothesis is supported by the finding that ACPA-IgG are highly agalactosylated, especially in RF-positive patients [10]. This may expose epitopes for RFs by inducing conformational changes in the IgG-Fc [21]. Using a biosensor system we found that serum RF showed comparable binding profiles when interacting with ACPA-IgG or non-ACPA-IgG. Among 41 RF-positive RA patients there were none with a dominant RF response preferentially binding to ACPA-IgG or non-ACPA-IgG. We conclude that ACPA-IgGs are not inherently bound better by RF than non-ACPA-IgGs. Still, RF-ACPA-IgG complexes may preferentially form over RF-non-ACPA-IgG complexes because of high local synovial production and the fact that a multiplicity of ACPAs binding to their citrullinated targets provides RFs with multiple Fc targets, facilitating a multivalent, high avidity interaction. Furthermore, since our SPR-setup measures total RF and our monoclonals are IgM-RF, it remains possible that IgA- or IgG-RF responses have a different fine-specificity.

The importance of Fc-galactosylation for binding of RF has been studied before with differing results. Soltys and colleagues [12] analyzed binding of synovial tissue derived monoclonal RFs to polyclonal IgG preparations of varying galactosylation status. They found that some RFs bound better to IgG preparations with lower galactosylation content whereas others bound independently of galactosylation content. Newkirk and colleagues [22] reported that monoclonal RFs bound equally to the Fc of polyclonal IgG from normal controls and polyclonal IgG with a lower galactosylation content from RA patients. Limited information is available on binding properties of polyclonal IgM-RFs. Imafuku et al. [11] found higher IgM-RF reactivity in 3/9 RA patients against agalactosyl IgG. The present study involved sera of more than 40 RF-positive RA patients and used defined targets to evaluate binding of RF to differently galactosylated IgG. The glycans on the recombinant monoclonal IgGs used as targets have been analyzed by mass spectrometry to accurately define their galactosylation percentage [16]. Using an SPRi-setup RF-IgG interactions were studied in real-time. A limitation of the SPRi is that the amount of IgG target coupled to the sensor during the spotting procedure can vary. Therefore, only sensor spots showing comparable signals with control anti-IgG-CH1 antibodies were

used for comparing RF-IgG and RF-ACPA interactions. Since frequent regeneration of the sensor can affect the coupled IgG-targets we ran a standard sample before and after the experiments with the patient sera (Supplementary figure 1) to ensure signal stability. Using these tools we found that the degree of galactosylation of the Fc domain does not appear to influence binding dynamics of polyclonal RF responses in RA patients. Furthermore, no patients showed a dominant RF response recognizing only IgG with low, normal or high Fc-galactosylation. Moreover, using two recombinant monoclonal IgM-RFs, one (mRF 61) that binds far away from the glycosylation site and one (mRF-AN) that binds much closer at the CH2-CH3 interface we show that potential conformational changes induced by regulating Fc-galactosylation did not influence RF binding.

While changes in Fc-glycosylation have been suggested to increase the pathological potency of ACPAs [23], a potential amplifying effect of RF through binding to the ACPAs does not seem to be dependent on these changes. This suggests that in the pathophysiology of RA amplification of ACPA-mediated inflammation by RF can occur *before* ACPAs acquire a more pro-inflammatory phenotype.

In conclusion, although recent literature suggests that interactions between RF and ACPA have a catalytic effect on inflammation, the present study demonstrates that RFs do not preferentially bind to ACPAs over non-ACPA-IgG or agalatosylated IgG over IgG with normal or high galactosylation.

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## SUPPLEMENTARY DATA

### *Galactosylation – Supplementary Methods*

#### Isolation of ACPA-IgG and non-ACPA-IgG from plasma

ACPA-IgG and non-ACPA-IgG were isolated from plasma of three RA patients by affinity chromatography (ÅKTA, GE Healthcare), as previously reported [1]. Before isolation, plasma samples were treated with 1.8 mg/ml EDTA and centrifuged for 10 min at 3000g. Supernatants were filtered over a 0.45 µm filter and loaded on biotinylated cyclic citrullinated peptide 2 (CCP2) or control (arginine) coupled HiTrap Streptavidin HP 1 ml columns (GE Healthcare). Flow through fractions containing the non-ACPA-IgG were collected and the ACPA-IgG fraction bound to the CCP2 column eluted with 0.1M glycine HCl, pH 2.5 and directly neutralized with 2M Tris. Flow through and elution fractions were further isolated to (non-) ACPA-IgG1, 2 and 4 with HiTrap Protein G and Protein A 5 ml columns (GE Healthcare). Afterwards, ACPA-IgG and non-ACPA-IgG samples were concentrated by centrifugal ultrafiltration (Amicon Ultra-15, 50K MWCO, Millipore) and desalted using Zeba desalt spin columns (7K MWCO, Thermo Scientific) according to the manufacturer's protocol. Protein concentrations were measured at 280 nm with a NanoDrop Spectrophotometer. The ACPA-IgG fractions showed high anti-citrulline reactivity in ELISA, whereas the non-ACPA-IgG fractions showed anti-citrulline reactivity at background (anti-arginine) levels (Supplementary Table S5.1).

#### Production of monoclonal antibodies with different galactosylation levels

Variable (V) genes for anti-human rhesus D (anti-D clone 19A10) heavy and light chain were sequenced from a single human B cell clone from a hyper immunized donor [2]. A single-gene vector containing anti-D or anti-2,4,6-trinitrophenol (TNP) IgG1 heavy- and kappa-light chain encoding sequences was cloned as described previously [3]. IgGs were produced by transient transfection of HEK-freestyle cells (Thermo Scientific) [3]. Glyco-engineering of IgGs and subsequent determination of the composition of Fc-glycans (Supplementary Table S5.2) by mass spectrometry was performed as described by Dekkers et al. [4].

#### Production of monoclonal rheumatoid factors

Two monoclonal IgM rheumatoid factors (mRFs) were produced. Variable heavy (VH) and variable light (VL) chain amino acid sequences for monoclonal RFs "RF 61" [5] and "RF-AN" [6] were retrieved from the Protein Data Bank [7], accession codes 2J6E and 1ADQ. Synthetic constructs coding for the variable domains and IgM and lambda constant domains were cloned into a pcDNA3.1 expression vector (Invitrogen) and



mRFs were produced under serum-free conditions (FreeStyle 293 expression medium; Invitrogen) by co-transfecting the heavy-chain- and light-chain-expressing vectors in HEK 293F cells using 293fectin according to the manufacturer's instructions (Invitrogen). The cells were cultured at 37°C, 8% CO<sub>2</sub>, shaking. Supernatant was harvested at day 5 and filtered over a syringe filter with a pore size of 0.45 µm. mRFs were isolated by loading the filtered supernatant onto a column with a Capture Select IgM affinity matrix (life technologies). After isolation, the mRF were analyzed with high performance size exclusion chromatography (HP-SEC) and the fraction containing the hexameric/pentameric mRF was separated from the monomer fraction with a Superose 6 10/300 GL column (GE Healthcare) for use in the experiments.

### Surface Plasmon Resonance imaging

For the Surface plasmon resonance imaging (SPRi) experiments the IBIS MX96 imager (IBIS Technologies, Enschede, the Netherlands) was used. ACPA-IgG and non-ACPA-IgG isolated from three patients and the three differently galactosylated monoclonal anti-D antibodies were spotted at 30nM, 10nM and 3nM spotting concentrations in 10mM MES (2-(N-morpholino)ethanesulfonic acid) buffer with 0.075% Tween-80, pH 6, onto pre-activated Easy2Spot G-type sensors (Ssens, Enschede, the Netherlands) containing active ester groups to covalently immobilize the target antibody, using a Continuous Flow Microspotter (Wasatch Microfluidics, Salt Lake City, USA). Serum samples, monoclonal RFs and control antibodies were diluted in PBS with 0.075% Tween-80 and 10mM EDTA. Samples were flowed over the sensor for 5 or 10 minutes in the association phase, followed by a 5-minute dissociation phase and regeneration of the sensor with a 12-second pulse of 10mM Glycine-HCl buffer, pH 2.4, with 1M NaCl and 0.075% Tween-80. Anti-CH1 llama antibody fragments (Thermo Fisher) were used to control for the amount of IgG coupled to the sensor-spots without interference by Fc-glycans.

### Serum samples

Serum samples from 46 early RA patients from the Amsterdam region were used in the SPRi experiments, 41 RF-positive and 5 RF-negative, determined by commercial RF assays. The RA patients fulfilled the 1987 criteria of the American College for Rheumatism (ACR) [8]. These patients had not been previously treated with disease modifying anti-rheumatic drugs (DMARDs). All patients signed informed consent forms for use of their serum samples. The RF-positive standard sample RELARES used in the experiments is a national reference serum with a defined IgM-RF level of 200 IU/ml [9].

## ELISAs

Target anti-TNP antibodies were diluted to 1  $\mu\text{g/ml}$  in 0.1% Tween 20–PBS (PBS-T) and incubated for 2 hours on Nunc MaxiSorp 96-well flat-bottom plates (Thermo Scientific) that had been coated overnight at 4°C with 10  $\mu\text{g/ml}$  TNP-ylated human serum albumin (HSA-TNP) in PBS. Plates were washed 5x with wash buffer (PBS/0.02%Tween-20) and incubated for 60 minutes with the mRFs diluted in PBS-T. RF binding was detected by incubation for 30 minutes with horseradish peroxidase (HRP)–conjugated mouse monoclonal anti-human IgM ( $\mu$ -chain–specific, MH-15, Sanquin) diluted to 0.33  $\mu\text{g/ml}$  in PBS-T and visualized by adding substrate solution containing 0.1 M NaAc pH 5.5, 10  $\mu\text{g/ml}$  tetramethylbenzidine (TMB) and 0.0003%  $\text{H}_2\text{O}_2$  (all from Merck, Germany) in distilled water. The reaction was stopped with 2M  $\text{H}_2\text{SO}_4$  and the optical density (OD) was read at 450 nm.

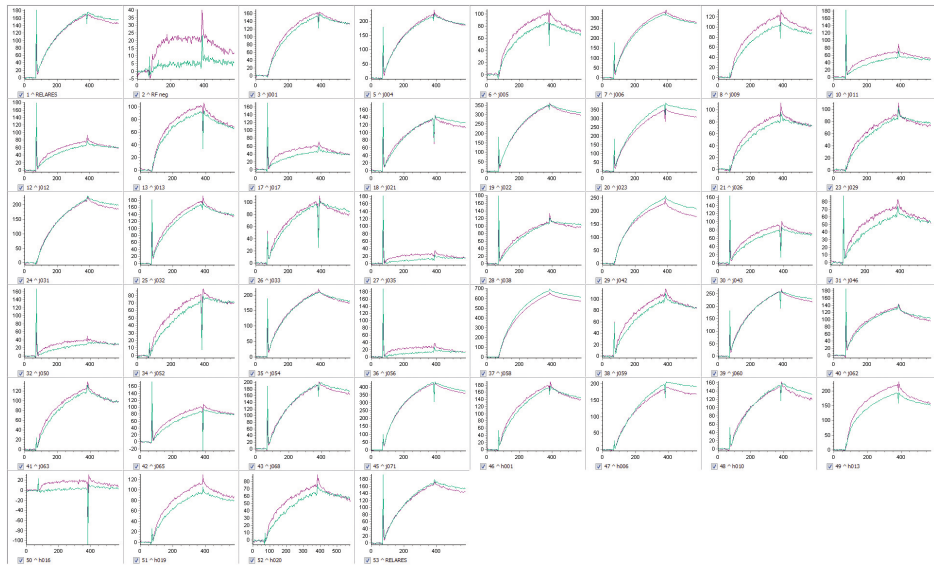
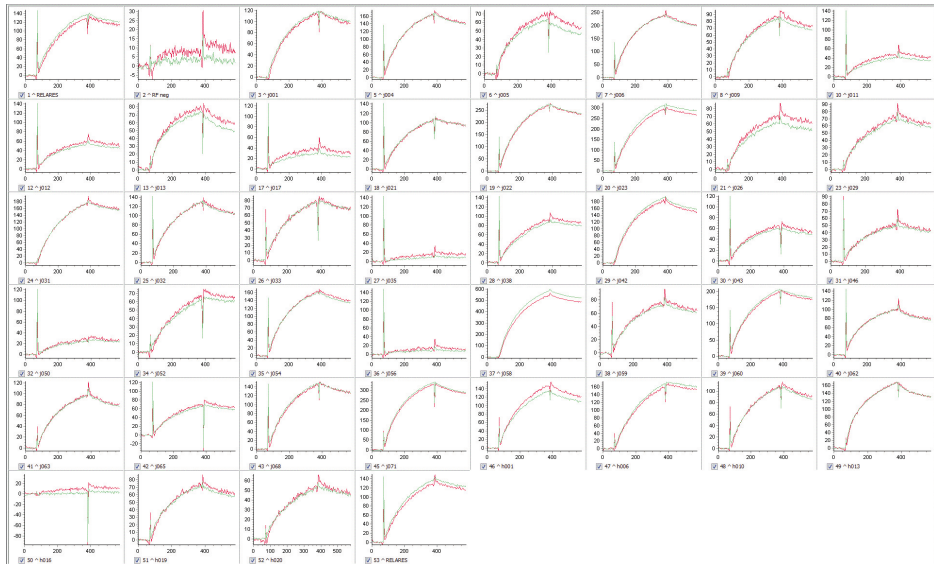
**Supplementary Table S5.1.** Anti-citrulline and anti-arginine reactivity of patient-derived ACPAs and non-ACPA IgGs

		IgG pt 1	IgG pt 2	IgG pt 3	ACPA pt 1	ACPA pt 2	ACPA pt 3
ACPA-IgG ELISA							
CITRULLINE	AU/ml	348.6	1029.0	487.2	12569.5	>8000	15178.8
ACPA-IgG ELISA ARGININE	AU/ml	679.4	na	1329.5	18.7	na	52.7
Protein concentration	mg/ml	69.1	53.1	76.0	1.1	4.0	1.1
Ratio CIT reactivity/mg protein	AU/mg	5.0	19.4	6.4	11747.2	>2000	13552.5

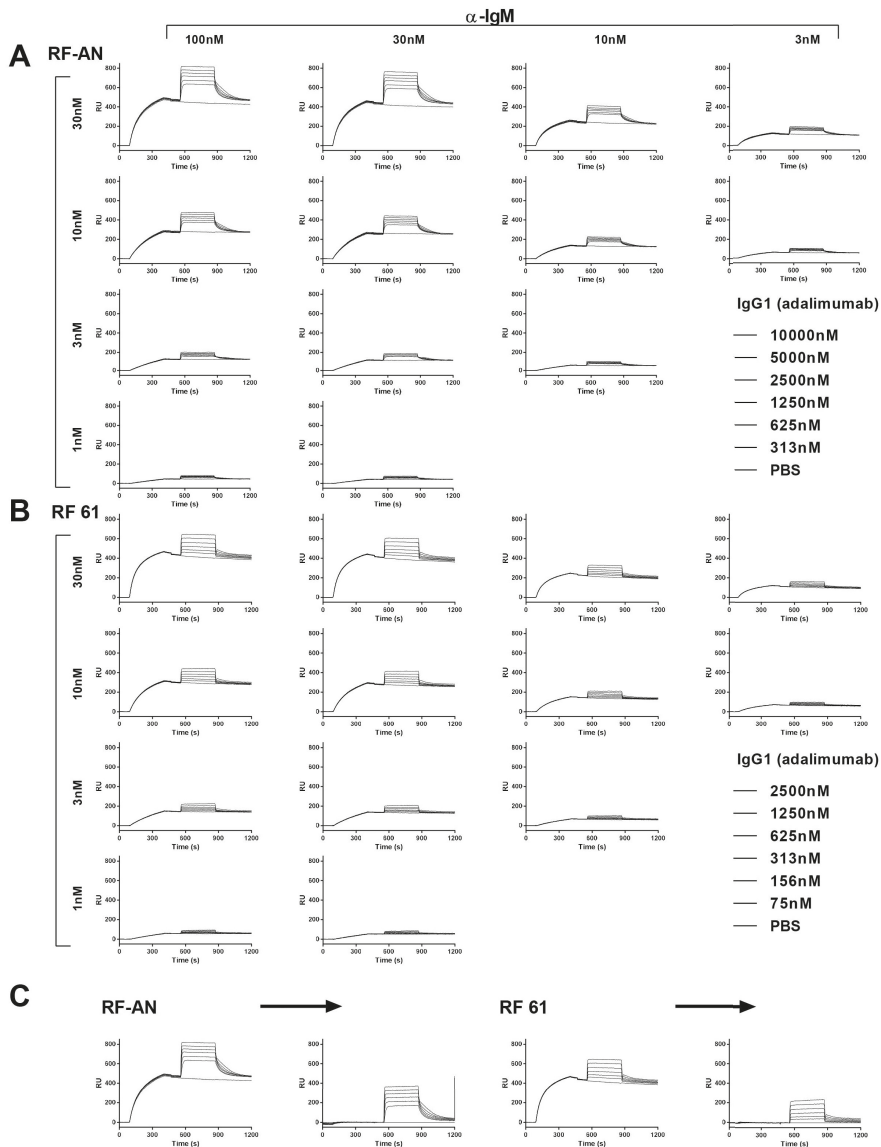
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**Supplementary Table S5.2.** Fc-glycan composition as determined by mass spectrometry

	Glycan content Fc domain in %			
	galactose	sialic acid	fucose	bisection
anti-D IgG1				
normal galactose	<b>29.0</b>	2.3	94.2	5.1
low galactose	<b>10.5</b>	0.9	94.4	3.3
high galactose	<b>84.8</b>	14.9	92.7	6.1
anti-TNP IgG1				
normal galactose	<b>19.3</b>	1.9	93.6	4.1
low galactose	<b>9.5</b>	1.7	88.0	4.7
high galactose	<b>82.9</b>	14.2	92.8	4.2

**A****B**

**Supplementary Figure S1: Serum RF binding kinetics to differently galactosylated IgG targets.** A) Sensorgrams showing association to and dissociation from IgG-targets with low (green lines) or high (purple lines) galactosylation for RF in 1:200 diluted sera from 41 RF-positive RA patients, of which 38 showed interpretable SPR-shifts. RF-positive reference serum RELARES was flowed over the sensor as the first and last sample to show stability of signals. B) As in (A), but with red lines representing binding kinetics with IgG-targets with normal galactosylation. Y-axes: SPR-shift in response units; X-axes: time in seconds.



**Supplementary Figure S2:** Affinity measurements for two monoclonal IgM rheumatoid factors. Recombinant monoclonal IgM rheumatoid factors, RF-AN (A) and RF 61 (B), were flowed at 30, 10, 3 and 1nM concentrations over a sensor to which mouse monoclonal anti-human IgM antibodies (MH15, Sanquin) had been coupled at 100-30-10-3nM. After a 5 minute association phase the flow was replaced with buffer and subsequently incubated with the depicted concentrations of IgG1 (adalimumab). Affinity constants were determined after subtracting the signal generated in the PBS condition (C) with SPRINT Software (IBIS technologies) by equilibrium analysis using Scrubber software (Biologic Software) with interpolation to  $R_{max}=100$  RU.

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