



ORIGINAL RESEARCH

Fc γ -receptor-IIIa bioactivity of circulating and synovial immune complexes in rheumatoid arthritisIvana Andreeva,¹ Philipp Kolb,^{2,3} Lea Rodon,¹ Norbert Blank,¹ Hanns-Martin Lorenz ¹, Wolfgang Merkt ^{1,4}

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ABSTRACT

Objective Previous technical limitations prevented the proof of Fc γ -receptor (Fc γ R)-activation by soluble immune complexes (sICs) in patients. Fc γ RIIIa (CD16) is a risk factor in rheumatoid arthritis (RA). We aimed at determining the presence of CD16-activating sICs in RA and control diseases.

Methods Sera from an exploratory cohort (n=50 patients with RA) and a validation cohort (n=106 patients with RA, 20 patients with psoriasis arthritis (PsA), 22 patients with systemic lupus erythematosus (SLE) and 31 healthy controls) were analysed using a new reporter cell assay. Additionally, 26 synovial fluid samples were analysed, including paired serum/synovial samples.

Results For the first time using a reliable and sensitive functional assay, the presence of sICs in RA sera was confirmed. sICs possess an intrinsic capacity to activate CD16 and can be found in both synovial fluid and in blood. In low experimental dilutions, circulating sICs were also detected in a subset of healthy people and in PsA. However, we report a significantly increased frequency of bioactive circulating sICs in RA. While the bioactivity of circulating sICs was low and did not correlate with clinical parameters, synovial sICs were highly bioactive and correlated with serum autoantibody levels. Receiver operator curves indicated that sICs bioactivity in synovial fluid could be used to discriminate immune complex-associated arthritis from non-associated forms. Finally, circulating sICs were more frequently found in SLE than in RA. The degree of CD16 bioactivity showed strong donor-dependent differences, especially in SLE.

Conclusions RA is characterised by the presence of circulating and synovial sICs that can engage and activate CD16.

INTRODUCTION

Rheumatoid arthritis (RA) is a rheumatic disease characterised by autoimmunity, with the focus of inflammation in the synovium. Systemic manifestations such as interstitial lung disease, osteoporosis and vasculitis can also occur. The pathophysiology of RA is not fully understood.¹ At least its seropositive form is in part mediated by autoantibodies such as rheumatoid factor (RF) and anticitrullinated

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Polymorphisms in the Fc γ -receptor (Fc γ R)-IIIa (CD16) gene are a risk factor in rheumatoid arthritis (RA).

WHAT THIS STUDY ADDS

⇒ A reporter cell assay demonstrates that RA is characterised by the presence of circulating and synovial soluble immune complexes that can engage and activate CD16.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The CD16 bioactivity of synovial soluble immune complexes may help to stratify patients with arthritis.
⇒ Closer biochemical characterisation of immune complexes with proven bioactivity at Fc γ Rs will lead to a better understanding of autoimmunity in RA.

peptide antibodies (ACPA).² Interestingly, autoantibodies are associated with extra-articular manifestations and erosiveness but can emerge prior to the onset of symptoms,^{3,4} indicating further factors driving arthritis than the mere presence of antigen-specific autoantibodies. Indeed, RA pathophysiology is a multistep process,⁵ and RF and ACPA poorly correlate with disease activity and treatment response and are not used as clinical biomarkers once the diagnosis of RA being established. As autoantibodies are nonetheless specific, for example, targeting citrullinated residues of several proteins, and associated with worse outcomes,^{6,7} an explanation for the incomplete pathogenicity of autoantibodies is needed. We hypothesise that this explanation may be found in regulatory or functional downstream processes aside antigen specificity or titres.

Through their Fc regions, IgG autoantibodies can engage Fc γ Rs such as Fc γ RIIIa/CD16. A function of human CD16 in ACPA-positive RA can be derived from

polymorphisms identified as genetic risk factors. Mouse models likewise propose a role of CD16 in autoantibody-mediated arthritis.^{8–12} Nevertheless, recently identified differences between murine and human CD16 warrants further study to understand the role of this FcγR in human disease.¹³

Importantly, monomeric IgG (auto)antibodies do not per se trigger CD16 activation—CD16 is a low affinity receptor requiring crosslinking and clustering for activation.^{14 15} As such, CD16 is mainly activated by immobilised IgG, cell-bound antibodies or soluble immune complexes (sICs).^{16 17} Most likely, CD16-mediated downstream effects of autoantibodies therefore depend on the three-dimensional space surrounding monomer autoantibodies, for example, on its organisation in sICs.

Indeed, preliminary studies from the 1980s suggested the presence of sICs in autoimmune diseases such as RA.^{2 18 19} Later on, several analyses of enriched protein complexes supposedly containing sICs using chromatography, precipitation and binding to C1q or protein G, for instance, suggested citrullinated antigens within sICs in plasma and synovial fluid in RA.^{20–22} On the functional level, it was suggested that sICs activate various cell types, including macrophages to produce tumour necrosis factor,²³ neutrophils to produce reactive oxygen species products²⁴ and osteoclasts to promote erosions.^{25 26} It should be mentioned, though, that in some of these studies on sIC function, immune complexes were artificially generated by adding antigens or performing heat aggregation.^{23 24 26} Other studies enriched sICs prior to in vitro analysis, which alters sIC concentration and cannot be differentiated from more artificial protein aggregates.²⁵ Newer studies used RNA-based cellular pathway signatures to indirectly illustrate the activation of joint neutrophils via IgG, but this may also result from deposited ICs.²⁴ Thus, the exact functions of untreated, native circulating and synovial sICs in vivo in RA remain elusive. In the past, detection of sICs in patient samples was limited by available technology. Previous assays designed to quantify sICs have failed to gain relevance in clinical application because of low sensitivity and specificity and low intermethod reproducibility. Furthermore, these methods are not able to measure sIC bioactivity, meaning their potential to activate FcγRs on expressing cells,^{18 27} leaving a gap in our understanding of the role of sICs.¹⁷ sIC bioactivity includes all parameters relevant to FcγR activation, such as sIC size, sIC concentration, IgG glycan profiles, IgG subclasses and IgG affinity.^{14 17 26 28} In RA specifically, the Fc glycosylation pattern influences the pro-inflammatory capacity of autoantibodies.^{10 29–32}

We have recently developed a novel cell-based reporter assay, which allows for the quantification of sIC bioactivity regarding host FcγR activation.^{17 33} This enabled us to demonstrate the presence of bioactive circulating sICs in SLE and severe COVID-19, where bioactivity was linked to disease severity.^{17 34} In addition, we were able to demonstrate a high CD16 bioactivity of synovial sICs in a pilot study in RA.³⁵ In the present study, we investigated

the CD16 bioactivity of circulating sICs in a large RA cohort and control cohorts. Furthermore, we compared local CD16 bioactivity in synovial fluid from patients with RA and patients without RA. Finally, we compared local with systemic CD16 bioactivity in paired and unpaired patient samples.

METHODS

Patients and materials

Written informed consent was obtained from every blood and synovial fluid donor. Clinical data were extracted from patient records. Data and samples were pseudomised. Patient characteristics are provided in [table 1](#) and online supplemental tables 1,2. Healthy controls (HCs) were volunteers without obvious signs of infection and without a known history of systemic autoimmune disease. These individuals were recruited within the environment of our university clinic and mostly employees or friends and relatives from our scientists. More characteristics of HCs are provided in [table 1](#).

Generation and culture of CD16+ BW5147 reporter cells

BW5147 reporter cells were kept in Roswell Park Memorial Institute medium supplemented with 10% fetal calf serum (FCS, Invitrogen), 4 mM glutamin (1/100, Sigma-Aldrich), 1x penicillin/streptomycin (Sigma-Aldrich), 1x sodium pyruvate (Gibco) and 0.1 mM beta-mercaptoethanol (Gibco). BW5147 reporter cells expressing human FcγRIIIA/CD16 ectodomains genetically fused to the transmembrane and cytosolic domains of mouse CD3-zeta were newly prepared for this study. Lentiviral transduction and selection were performed as described previously.^{17 34 35} In brief, chimeric FcγR-mCD3-zeta constructs encoded on a lentiviral packaging vector and supplementing vectors providing the VSV gag/pol and VSV-G-env proteins were transfected into a 293T packaging cell line at roughly 70% density in a 10 cm dish format. After 3 days, supernatant was collected, filtered (0.45 μm) and incubated with parental BW5147 cells (3.5 mL of supernatant on 10⁶ target cells) followed by culture expansion and antibiotics pool selection using complete medium supplemented with 2 μg/mL of puromycin (Sigma) over a 1-week culture period. Parental cell selection served as a point of reference. Cell sorting was performed at the Lighthouse core facility of the University Hospital Freiburg using receptor staining (clone 3G8, BD Pharmingen, PE-conjugated). Receptor expression was evaluated regularly via flow cytometry using a fluorescein isothiocyanate (FITC)-conjugated antihuman CD16 antibody (clone 3G8, BioLegend). Parental cells served as a point of reference and staining using FITC-conjugated antihuman CD55 (Cat. 311305; BioLegend) served as isotype control. Flow cytometry was performed on a BD LSR II flow cytometer. Data were analysed using FlowJo software V.10.7.1.

FcγR reporter activation assay

FcγR activation was measured using a previously described cell-based assay.^{17 34 35} Briefly, 2×10⁵ reporter

Table 1 Patient characteristics (validation cohort)

	HC	PsA	RA	SLE
Number of samples	31	20	106	22
Per cent female	58%	55%	72%	100%
Age, mean	35	55	61	36
Age, range	(23; 76)	(32; 74)	(19; 88)	(19; 68)
Per cent RF positive	nd	10%	70%	nd
Per cent ACPA positive	nd	10%	75%	nd
Patients on corticosteroids	nd	5	37	14
Mean steroid dose	nd	7.6 mg	6.4 mg	9.2 mg
Patients on DMARDs	nd	14	71	22
Methotrexate	nd	8	43	3
Leflunomide	nd	3	25	0
Sulfasalazine	nd	4	3	0
Hydroxychloroquine	nd	0	1	17
Ciclosporin	nd	0	0	1
Mycophenolate mofetil	nd	0	0	8
Azathioprine	nd	1	2	4
Patients on biologicals	nd	7	24	6
Tumour necrosis factor inhibitors	nd	4	14	0
Anti-IL-6 antibody	nd	0	5	0
Anti-IL-12/23 antibody	nd	1	0	0
Anti-IL-17A antibody	nd	2	0	0
Abatacept	nd	0	4	0
Belimumab	nd	0	0	4
Rituximab/Obinutuzumab	nd	0	1	2
Patients on JAK inhibitors	nd	0	20	0

Numbers represent absolute numbers except for the the gender row; numbers in the latter represent the percentage of female patients. Of note, two female patients (aged 36 years and 66 years) were punctured twice. DMARDs, disease-modifying antirheumatic drugs; HC, healthy control; IL, interleukin; JAK, Janus kinase ; nd, not determined; PsA, psoriatic arthritis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

cells expressing human FcγRIIIA/CD16 or parental cells lacking receptor expression were incubated with graded amounts of patient sample in a 96-well format in a total volume of 100 μL. Incubation on immobilised human intravenous IgG served as positive control for receptor activation (20 μg/mL Octagam, Octapharma on NUNC MaxiSorp plate at 4°C, 16 hours incubation). Incubation with medium alone served as a negative control for receptor activation. Incubation was performed in a 96-well ELISA plate (NUNC MaxiSorp) pretreated with phosphate-buffered saline (PBS)/10% FCS (v/v) for 1 hour. Reporter cell mouse interleukin (IL)-2 secretion was quantified after 16 hours of incubation via anti-IL-2 ELISA (Mouse IL-2 DuoSet, R&D). IL-2 concentrations normalised to the intra-experimental negative control via subtraction and area under the curve (AUC) index values were calculated from titrations (GraphPad Prism). Alternatively, reporter cells were analysed for mouse CD69 expression using a BD LSR II flow cytometer. Staining

was performed in PBS/2% FCS using an APC-labelled antimouse CD69 antibody (Clone H1.253, BioLegend). Armenian Hamster IgG (BioLegend) served as a staining control. Additionally, PE-labelled antihuman IgG Fc (BioLegend) was used to control for the binding of human IgG to mouse reporter cells. Data were analysed using FlowJo software V.10.7.1.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software V.9 and V.10. P values <0.05 were considered significant and were interpreted descriptively. Unless stated otherwise, normal distribution of the data was not assumed, and tests were two-sided. The specific tests used are indicated in the figure legends. Graphics were in part created with BioRender.com. If not otherwise indicated, outliers were identified and excluded using ROUT.

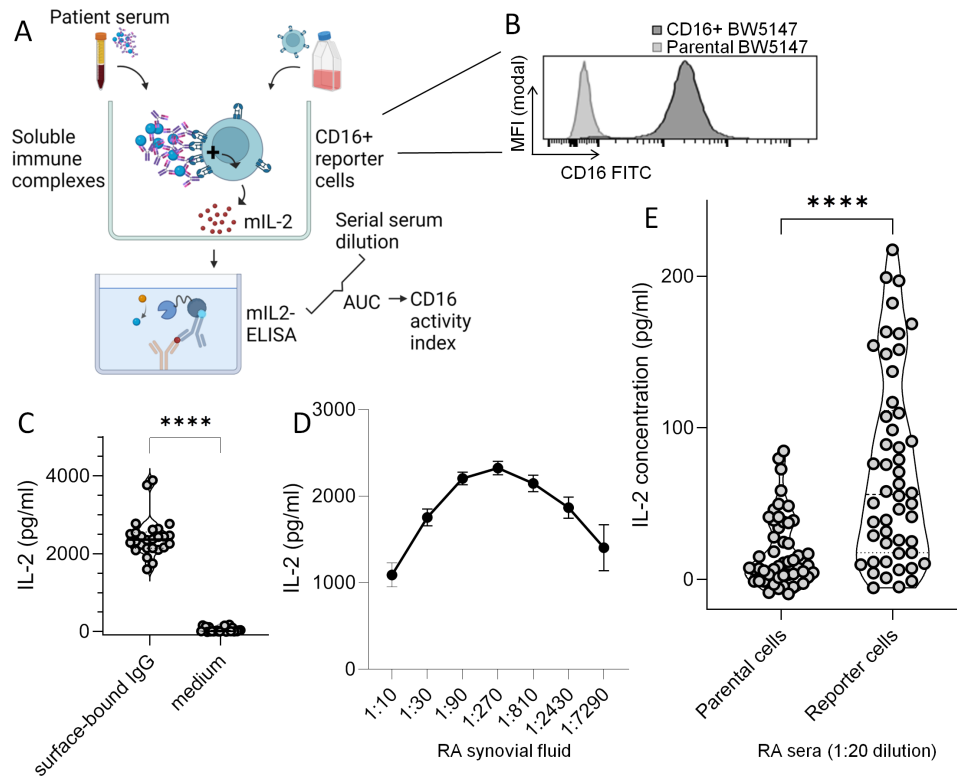


Figure 1 Evaluation of CD16 activation by surface-bound IgG and soluble immune complexes in patient samples using CD16+ reporter BW5147 cells. (A) Graphic experimental design. Patient samples (serum or synovial fluid) was added to CD16+ reporter cells (mouse BW5147 thymoma cells transduced with a construct containing extracellular human CD16) and incubated for 16 hours. CD16 engagement was measured by quantification of interleukin (IL)-2 secretion using ELISA. (B) Expression of CD16 on non-transfected parental and CD16+ reporter cells. The histogram shows an overlay of parental BW5147 cells and successfully transduced CD16+ reporter cells as determined by flow cytometry. (C) IgG complexed to plastic surface activates CD16+ reporter cells. IL-2 concentration produced by CD16+ reporter cells cultured overnight in intravenous immunoglobulin (IVIg)-coated wells across 26 experiments; IVIg-coated wells and culture medium were used as positive and negative controls in experiments with patient samples throughout this study. (D) Soluble immune complexes (sICs) activate CD16+ reporter cells. Synovial fluid from a patient with rheumatoid arthritis (RA) known to contain sIC³⁵ was tested in all n=26 experiments with patient sera as an additional positive control. Error bars indicate SEM. With stable CD16 expression on reporter cells, serial dilution of serum lead to a bioactivity-response curve that mimics the Heidelberger-Kendall curve, which describes the size of immune complexes depending on antigen/antibody ratios. (E) Circulating immune complexes (cICs) activate CD16+ reporter cells. In an exploratory study, parental and CD16+ reporter BW5147 cells were stimulated with serum samples from patients with RA (n=50) at a dilution of 1:20. The violin plot depicts individual patients using the average IL-2 concentration from technical duplicates. Statistical analysis with Wilcoxon matched-pairs test. ****P (two-tailed)<0.0001.

RESULTS

As outlined in the ‘Introduction’ section, there is compelling evidence of the importance of autoantibodies and CD16 (FcγRIIIA) in the pathophysiology of RA. Still, the presumable functional link between autoantibodies and the activation of the low affinity receptor CD16 via the formation of sIC remains to be proven. At present, it is unclear whether sIC in RA are indeed bioactive and can engage FcγRs such as CD16.

CD16+ bioactivity measurement identifies systemic sICs in patients with RA

To overcome drawbacks of previously available methods, we applied a new CD16 reporter cell model that directly determines the bioactivity of body liquids at the CD16 receptor (figure 1A,B). The CD16+ reporter cells produce mouse IL-2 on binding of complexed human IgG to CD16. Thus, they detect sICs in liquids such as synovial

fluid and serum without necessitating isolation steps and allow for conclusions of both the presence of immune complexes and their bioactivity in these liquids.^{17 34 35}

Immobilised IgG is used as a positive control in all the experiments performed with patient liquids in this study (figure 1C). Culture medium served as a negative control. As shown in our previous study, CD16+ reporter cells can detect sICs in synovial fluid.³⁵ As we slightly adapted the protocol, we confirmed the bioactivity of the same synovial fluid samples as used in the previous study.³⁵ We employed this synovial fluid sample as a second positive control throughout this study (figure 1D). Interestingly, serial dilution of this synovial fluid sample resulted in a bioactivity curve that resembled the Heidelberger-Kendall curve (figure 1D). The dilution of patient samples thus affects outcome of the reporter cell model in a complex, non-linear way.

Wondering whether CD16-activating sICs may be also be found in circulation, we conducted an exploratory study using a single dilution (1:20) to screen banked sera from patients with RA (n=50). As shown in [figure 1E](#), RA sera activated CD16+ reporter cells, while the parental cells lacking CD16 were not activated.

Taken together, these data show that CD16+ reporter cells can detect IgG complexed on surfaces or in sICs. The results from the exploratory study suggest the presence of circulating sICs in sera of patients with RA with an intrinsic capacity to activate CD16.

Cohort analysis reveals significant elevation of systemic sICs in RA

Having observed bioactivity of RA serum on the CD16 receptor in the exploratory screening study using a 1:20 dilution ([figure 1D](#)), we next compared the bioactivity of serum from patients with RA with HCs as well as with control rheumatic diseases. Psoriasis arthritis (PsA) is an important differential diagnosis of RA lacking IgG autoantibodies, while systemic lupus erythematosus (SLE) is associated with IgG autoantibodies targeting multiple antigens. SLE can be regarded as a positive control for the presence of circulating sICs, as we had discovered bioactivity at the CD16 receptor in our previous study.¹⁷ Patients characteristics are described in [table 1](#). As repeated experiments with synovial fluid indicated variable bioactivity at CD16¹⁷ depending on the degree of experimental dilutions ([figure 1D](#)), we performed the reporter cell assay using serial dilutions of serum from SLE, RA, HCs and PsA ([figure 2A](#)). Circulating sICs in SLE were confirmed to possess high CD16 bioactivity ([figure 2B](#)). Similar to what was observed with sIC-containing synovial fluid, serial dilutions of SLE serum resulted in a bioactivity curve indicating an influence of sIC concentrations on CD16 activation on reporter cells, with an optimal activation at 1:90 dilutions. In contrast to SLE, serum from RA, HC and PsA showed much lower activation through CD16. RA serum reached an optimal CD16 activation at a lower dilution (1:30) as compared with SLE ([figure 2B](#)).

For statistical analysis, we calculated the AUC of these serial dilutions, resulting in the 'CD16 activation index' ([figure 2C](#)). CD16 activation was variable within groups. While RA had a significantly higher index compared with HC and PsA, we also noted CD16 activation in several HCs.

Diagnostic autoantibody tests frequently use dilutions between 1:80 and 1:100. We used the 1:90 dilution to determine the percentage of individuals within each group that were tested positive for CD16 bioactivity ([figure 2D](#)). As a cut-off for being positive, we used a value of '1' pg/mL IL-2 (nota bene: each IL-2 value refers to the result after subtraction of the background IL-2 concentration in wells with culture medium lacking any human serum). This cut-off was chosen, as serum from RA, HC and PsA reached values below 1 in very high dilutions ([figure 2B](#)). This analysis confirmed the

presence of CD16 bioactivity in about every fourth HC and patient with PsA. It was more frequent in RA and SLE (54.7% and 81.8%). Fisher's exact tests confirmed that CD16 bioactivity was significantly more frequent in RA compared with HC and PsA, but less frequent compared with SLE (p=0.007, 0.026 and 0.030, respectively).

As the study had been performed during the COVID-19 pandemic, we excluded a significant impact of COVID-19 vaccination on the CD16 activation index ([figure 2E](#)).

A comparison with clinical data indicated no correlation of clinical parameters on CD16 activation index, including age, sex, duration of the disease, the concentration of RF and ACPA, disease-activity index-28 (DAS-28) or C reactive protein (CRP) levels ([table 1](#), [figure 2F](#) and online supplemental figure S1). No association to specific therapeutic agents was found either (online supplemental figure S2).

In summary, the validation cohort confirmed that circulating sICs possess CD16 bioactivity in RA. CD16 bioactivity is more frequently observed in RA and in SLE compared with HC and PsA. While overall systemic CD16 bioactivity can be very high in patients with SLE, it is rather low in RA, HC and PsA, however, with an important interindividual variability.

CD16 bioactivity in synovial fluid can identify types of arthritis

In the previous section, we reported an increased frequency of bioactive sIC in RA serum, yet in low quantities in most cases and without correlation to clinical parameters. As it had been reported that autoantibodies and immune complexes accumulate in joints in RA, we next analysed CD16 bioactivity of RA synovial fluid on several levels ([figure 3A](#)).

The comparison between serum and synovial fluid from different patients with RA indicated significantly higher bioactivity of synovial sIC compared with circulating sICs ([figure 3B](#)).

Synovial fluid from RA possessed a significantly higher CD16 bioactivity than synovial fluid from patients without RA (non-RA) ([figure 3C](#)). In the non-RA synovial fluid group, two samples were recognised as outliers using the ROUT method—these samples stemmed from the same patient who underwent arthrocentesis at two different time points and had high levels of antinuclear antibodies (ANAs) indicating that ANA-positive arthritis is associated with synovial sICs. When eliminating these outlier values from the group and creating a receiver operator curve (ROC), an AUC of 0.93 was obtained ([figure 3D](#)). This value of 0.93 indicated that synovial sICs could be used to discriminate RA from non-RA. Alternatively, it indicates that the reporter cell model could be used to group distinctive causes of arthritis. The latter interpretation allows re-allocation of the ANA+ outliers to the 'RA' group resulting in a combined group of autoantibody-positive arthritis that can be separated from a group of autoantibody-negative arthritis by determining synovial CD16 bioactivity. The ideal cut-off value resulting from ROC was a CD16 activation index of 440, yielding a

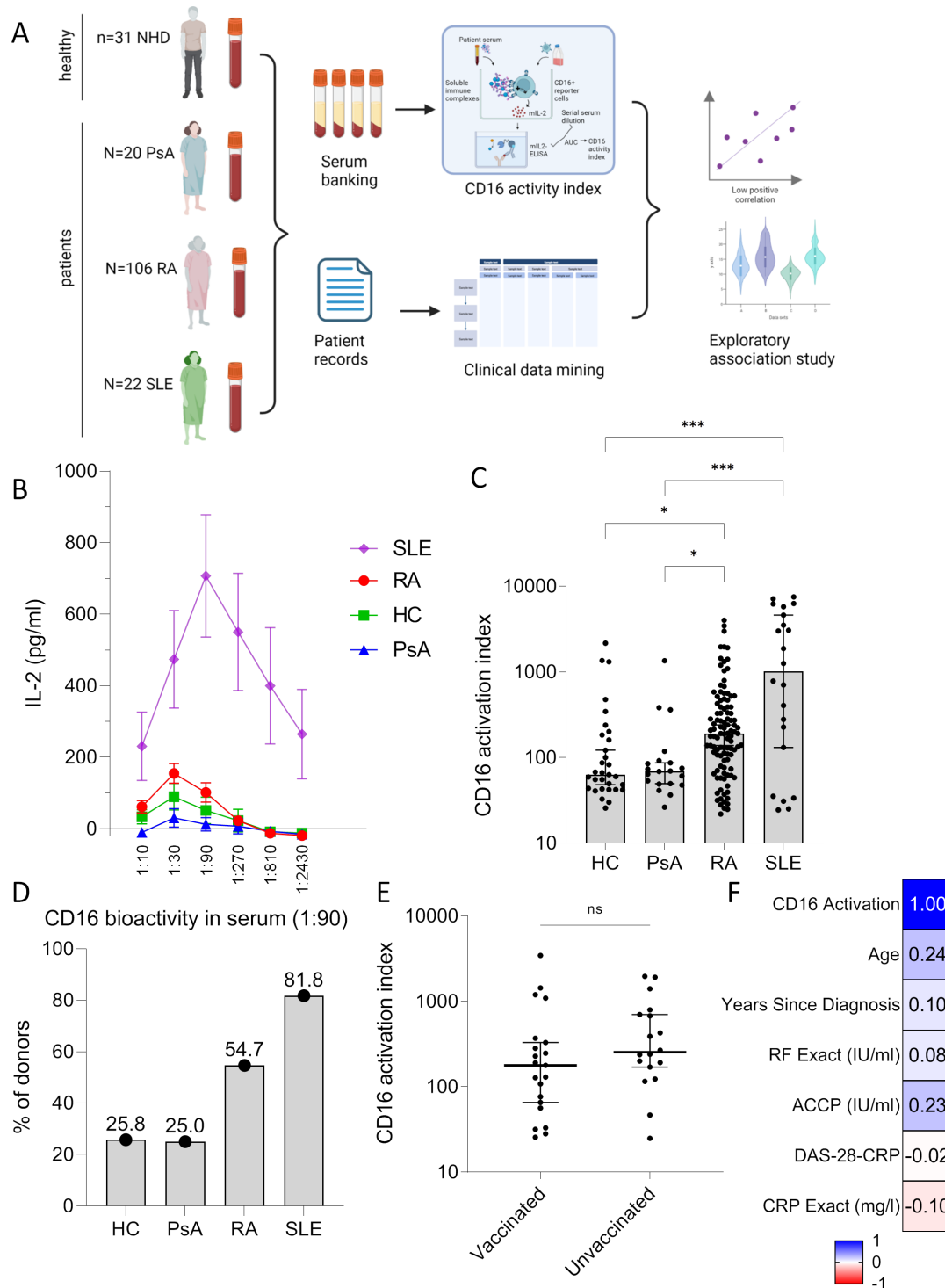


Figure 2 Detection of bioactive soluble immune complexes in serum from patients with rheumatoid arthritis (RA) using CD16 activity index. (A) Graphic experimental design. Serum samples from the following groups were enrolled: healthy controls (HC; n=31), patients with psoriatic arthritis (PsA; n=20), RA (n=106) and systemic lupus erythematosus (SLE; n=22). (B) Interleukin (IL)-2 secreted by CD16+ reporter BW5147 cells cultured with serial dilutions of serum overnight was determined by ELISA. (C) CD16 activation index (=area under the curve of (IL-2 concentration of the sample–IL-2 concentration in medium control)) induced by serum. Statistical analysis performed with Kruskal-Wallis test, significant post-tests are indicated by stars. (D) Percentage of individuals within each group tested positive for CD16 bioactivity. As a cut-off, a value of ‘1’ pg/mL IL-2 was chosen, as serum from RA, HC and PsA yielded values below 1 in very high dilutions (see B). (E) CD16 activation index in patients with RA who were vaccinated (n=21) and unvaccinated (n=18) against the novel SARS-CoV-2 virus. Sample size is limited to patients with RA whose serum was collected after start of the SARS-CoV-2 pandemic in 2020. Graph depicts individual patients and medians. Statistical analysis performed with Mann-Whitney U test. (F) Correlation matrix showing Spearman's r for comparisons with CD16 activation index. CRP, C reactive protein; DAS-28, disease-activity index-28; ns, not significant; RF, rheumatoid factor. *P<0.05; ***p<0.001.

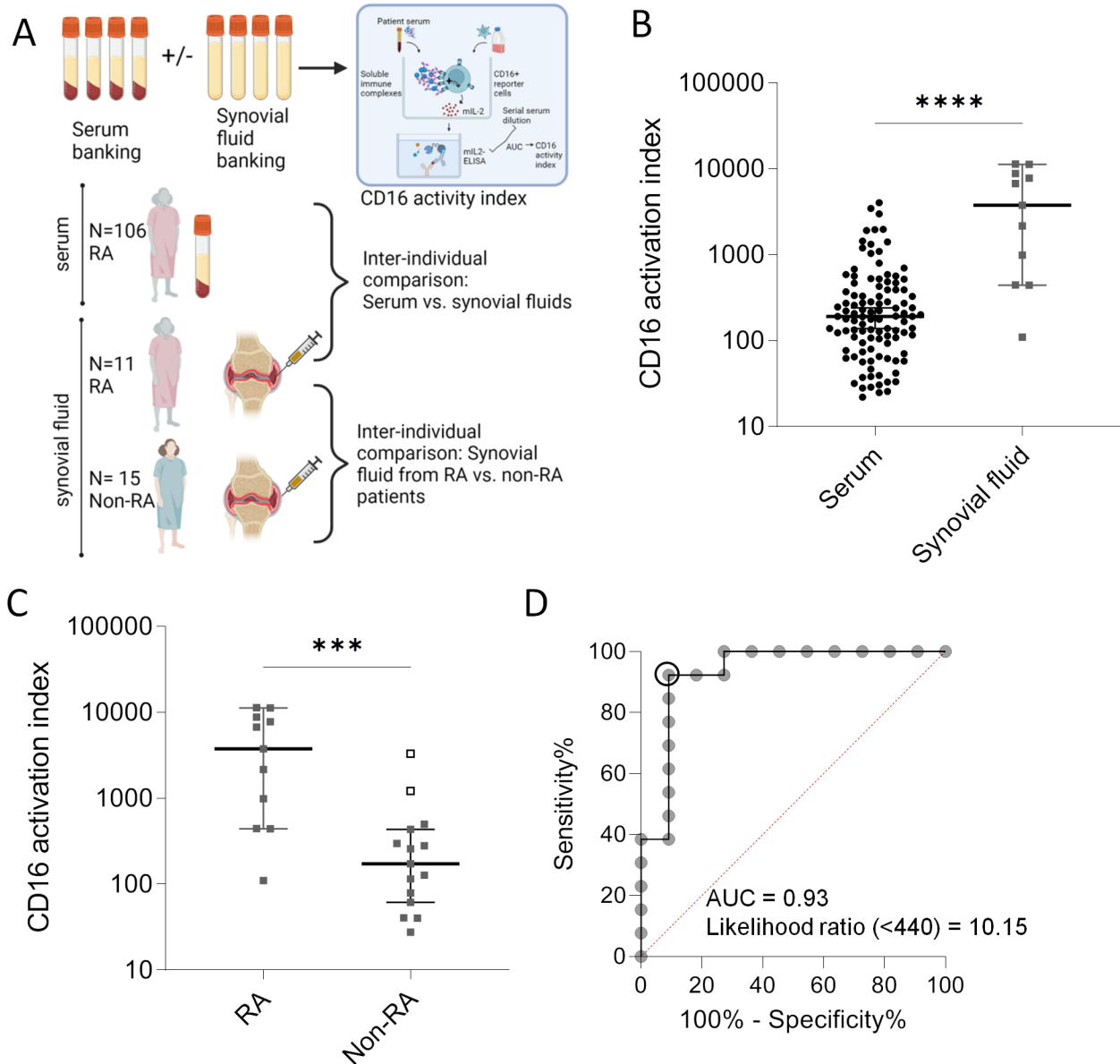


Figure 3 CD16 bioactivity in synovial fluid in patients with arthritis. (A) Graphic experimental design. Unpaired serum and synovial fluid samples used to determine CD16 bioactivity in these liquids using the reporter cell model. (B) Comparison of unpaired rheumatoid arthritis (RA) serum (n=106) and synovial fluid (n=11) samples. The y-axis represents the CD16 activation index (area under the curve (AUC) for the interleukin (IL)-2 concentrations of six dilution steps of each sample). Graph depicts individual patients as well as medians and 95% CI. Statistical analysis with Mann-Whitney U test. ****P (two-tailed)<0.0001. (C) Comparison of RA (n=11) and non-RA (n=15) synovial fluid samples. Non-RA diagnoses include osteoarthritis, psoriatic arthritis, spondyloarthritis, immune-related adverse event-associated arthritis, gout, polymyalgia rheumatica. For patient details, see online supplemental tables 1–3. Two patients without RA (non-RA) were punctured twice. The two empty squares correspond to the same patient included at two different time points suffering from juvenile idiopathic oligoarthritis; this patient had high antinuclear antibodies (ANA 1:5120) and was identified as an outlier using the ROUT method. Graph depicts individual patients as well as medians and 95% CI. Statistical analysis with Mann-Whitney U test. ***P (two-tailed)=0.0005. (D) Receiver operator curve resulting from data shown in (C); the outliers were not included. Using CD16 activation index of <440 as a threshold yielded in a likelihood ratio of 10.15.

likelihood ratio of 10.15 (sensitivity of 92.3% and specificity of 90.9%) (figure 3D).

The comparison between paired serum and synovial fluid samples from the same RA donors (n=3, all were seropositive) confirmed that synovial fluid possessed a significantly higher CD16 bioactivity than serum (figure 4A and B). This was the case despite relatively

high serum CD16 bioactivity in these patients, the serial dilution curves of which, additionally, described a bioactivity curve (figure 4B). Paired samples from non-RA showed no differences between synovial fluid and serum, but were anyway negative regarding CD16 bioactivity (figure 4B).

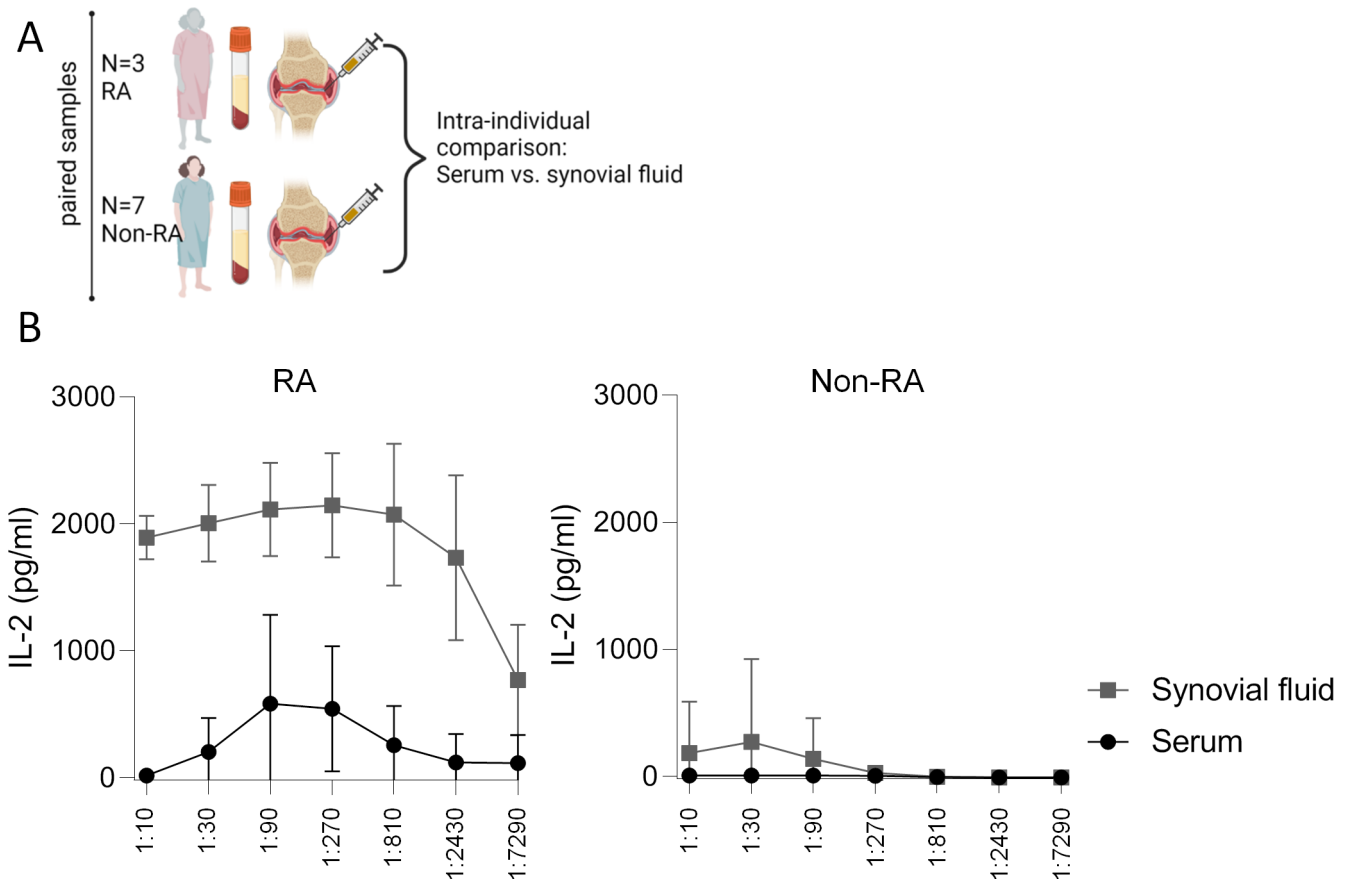


Figure 4 CD16 bioactivity in synovial fluid versus serum in patients with arthritis. (A) Graphic experimental design. Paired serum and synovial fluid samples were sampled and used to determine CD16 activation indices in these liquids using the reporter cell model. (B) Comparison of paired serum and synovial fluid samples from patient with seropositive rheumatoid arthritis (RA, $n=3$, left) and control diseases ($n=7$ patients without RA (non-RA) with joint effusions including two patients with psoriasis arthritis and each one patient with reactive arthritis, juvenile idiopathic arthritis, spondyloarthritis and immune-related adverse event arthritis). The graphs show mean and SD of interleukin (IL)-2 concentration produced by CD16+ reporter cells in serial dilution experiments. Serum and synovial fluid from non-RA control diseases did not lead to statistically different CD16 activation, while two-way repeated measures analysis of variance confirmed significant differences between RA serum and RA synovial fluid ($p=0.035$) and also between dilution steps ($p=0.007$).

As reported above, we observed no correlation between CD16 activation index in serum and clinical parameters in 106 patients with RA. In contrast, CD16 activation index determined in synovial fluid correlated with serum titres of RF (Spearman's $r=0.61$, $p<0.05$) and anti-CCP antibodies ($r=0.66$, $p<0.05$), while no correlation with age ($r=0.20$) and synovial cell number ($r=0.26$) could be detected.

Together, these data show high CD16 bioactivity in synovial fluid from RA, with significant differences compared with serum and patients with non-RA arthritis. The data further suggest that CD16 bioactivity may be used to distinguish different forms of arthritis.

Confirmation of sIC-induced activation of CD16+ reporter cells and loading with human IgG-Fc by flow cytometry

The reporter cell model demonstrated the bioactivity of immune complexes in serum and in synovial fluid. While these results were based on the secretion of IL-2 by activated reporter cells, we next intended to confirm the reporter cell activation and the binding of human IgG by

flow cytometry (figure 5A). As previously established, Fc γ R reporter cell activation can also be measured via upregulation of surface mouse CD69.¹⁷ Incubation of synovial fluid or RA serum containing sICs with reporter cells (in 1:90 and 1:30 dilutions in culture medium, respectively) shows significant CD69 upregulation (figure 5C and E) paired with loading of reporter cells with human IgG (figure 5B and D).

Using our model, we also addressed the question if pro-inflammatory IgG modifications in SLE such as de-sialylation are sufficient to activate CD16 even in the absence of sIC formation. To this end, we compared sera from patients with active SLE with a low sIC index (sIC-) with sera from patients with a high sIC index (sIC+) with regard to overall IgG binding to the reporter cells. As shown in figure 5F,G, both sIC- and sIC+ SLE sera provided IgG for efficient binding to CD16+ reporter cells. While there was a tendency of higher binding of IgG in sIC+ SLE sera, the patient number was too low to confirm mathematical significance. Importantly, despite

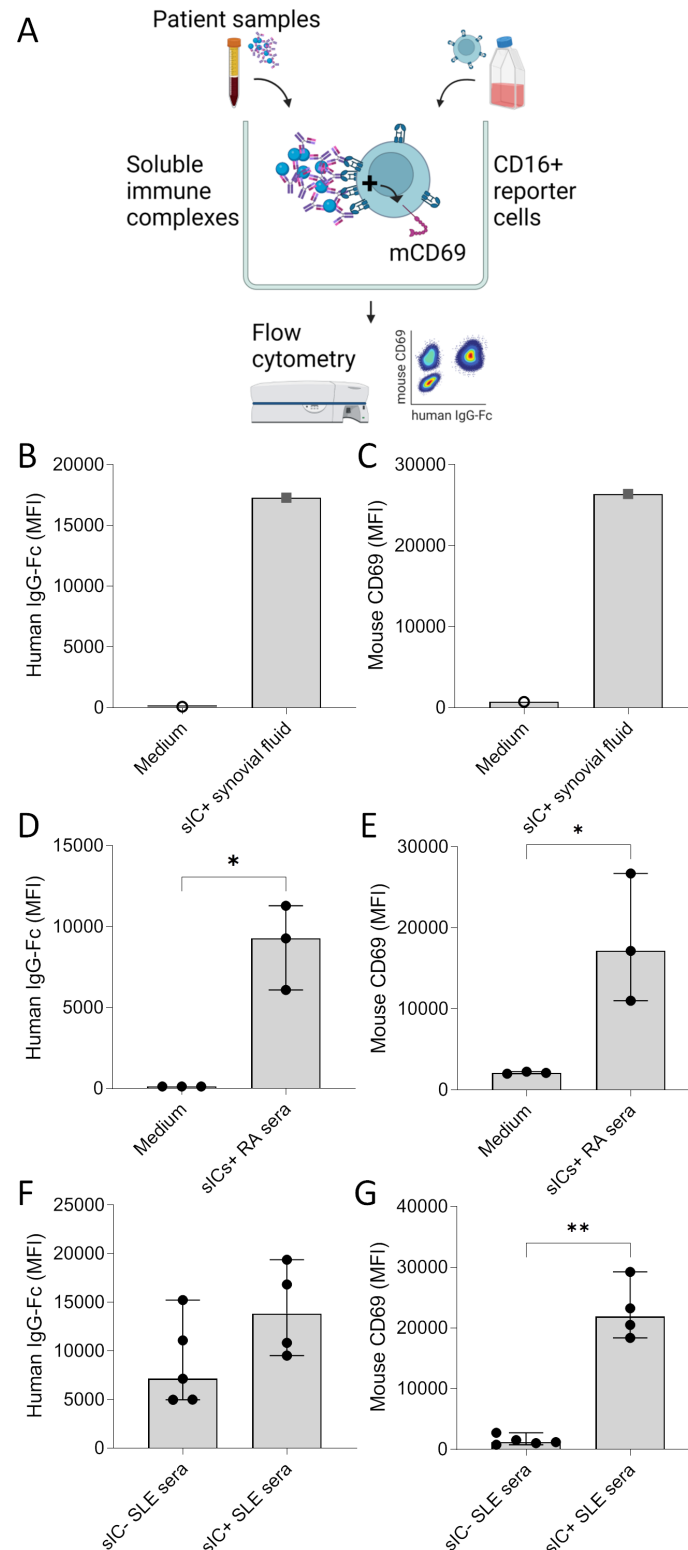


Figure 5 Confirmation of soluble immune complex (sIC)-induced activation and loading with human IgG-Fc on CD16+ reporter cells by flow cytometry. (A) Graphic experimental design. CD16+ reporter cells were incubated for 16 hours with medium and synovial fluid or serum and analysed by flow cytometry. (B/D/F) Mean fluorescence intensity (MFI) of reporter cells stained with a PE-labelled antihuman IgG Fc antibody after incubation with soluble immune complex-containing (sIC+) synovial fluid from a seropositive RF+ACPA+ patient with rheumatoid arthritis (RA) ($n=1$, figure 1D) and medium as negative control ($n=1$) (B), with sIC+ serum from patients with RA ($n=3$, selected based on CD16 bioactivity as shown in figure 2), and medium as negative control ($n=3$) (D), and with sera from patients with systemic lupus erythematosus (SLE). SLE sera were selected based on their CD16 bioactivity (figure 2) to contain sICs (sIC+, $n=4$) or not (sIC-, $n=5$) (F). (C/E/G) MFI of reporter cells stained with an APC-labelled antimouse CD69 antibody. The experiments were identical to the B/D/F, respectively, with C corresponding to B, E to D and G to F. Statistical analysis performed with one-tailed Mann-Whitney U tests. * $P < 0.05$, ** $p < 0.01$.

IgG loading of reporter cells by both sIC⁻ and sIC⁺ SLE sera, only sIC⁺ sera induced significant CD69 upregulation. This confirms that monomeric SLE IgG can bind to CD16 without inducing CD16 activation

Taken together, flow cytometry confirmed the binding of human IgG to the reporter cells as well as the activation of reporter cells by sICs. It also confirmed the non-functional binding of monomeric IgG to CD16 and highlighted the importance of complexed forms of IgG in inducing cell activation through CD16 signalling.

DISCUSSION

The functional proof of circulating and synovial sICs in RA is the key finding of our study. This provides a missing piece in the chain of events in RA pathophysiology, linking the serological products resulting from adaptive immunity to FcγR(IIIa)-dependent innate immunity.

The highly sensitive reporter cell model we applied¹⁷ defined sICs via the functional engagement by CD16 and allowed the direct measurement of sICs in patient samples. This enabled a unique approach to the global characterisation of sICs in RA, integrating previously neglected but relevant factors such as complex size and IgG glycan profile. Another advantage of this method is that it only detects multimeric soluble ICs and is not confounded by monomeric IgGs in clinical samples. A different assay measuring CD16 bioactivity has recently been introduced, but not used to test serum or plasma from patients.³⁶

We find that serial dilution of patient serum samples results in non-linear response curves with decreasing CD16 bioactivity in low dilutions (figures 1D and 2B). While proof is missing, we hypothesise that these curves—resembling the Heidelberg-Kendall precipitation curve describing IC size depending on antigen/antibody ratios—are a surrogate of varying sizes of the ‘super-complexes’ composed of sICs and FcRs. Given the stable expression of FcγRs on the reporter cell surface, increasingly low dilutions potentially surpass an activation plateau after which sICs saturate the FcγRs, preventing receptor cross-linking. To the best of our knowledge, we describe this finding for the first time, but further control experiments, for example, by using serum-free media, are warranted. Alternatively, we hypothesise that reporter cells experience activation-induced exhaustion or inhibition by other serum factors which may cross-react with the mouse thymoma reporter cell line. The latter is, however, less likely as peak mouse IL-2 secretion was found at the second lowest dilution in RA (figure 2B). Undefined serum factors may also explain the patient-dependent background IL-2-production by parental reporter cells devoid of FcγRs. We conclude that patient samples should therefore be measured in graded amounts to detect sICs across a wide range of concentrations.

We find sICs with high CD16 bioactivity in RA serum and synovial fluid. This finding is of high clinical relevance given the role of CD16 in RA suggested by genetic

risk association studies that were strengthened by mechanistic animal models.^{11 12} We confirm the accumulation of sICs in joints in RA.¹⁹ In this study, we expanded on this using a functional model. Beyond that, the comparison of RA with non-RA synovial fluid samples including osteoarthritis, psoriatic arthritis, HLA-B27-positive spondyloarthritis, immune-related adverse event-associated arthritis showed intriguing inter-disease differences in the CD16 bioactivity of synovial sICs. Non-RA diseases lacked detection of sICs in our assay. This finding is in line with previous knowledge on the response to B cell-targeting therapies such as rituximab, an anti-CD20 antibody with efficacy in RA and SLE but not in PsA or other non-RA arthritides.³⁷ However, the non-RA group in which synovial fluid was analysed was heterogeneous, precluding general statements of local immune complexes in individual diseases within this group. Whether a CD16-sICs-based categorisation of arthritis could assist in clinical decision-making needs to be investigated in the future.

In total, sera from over 150 patients with RA were analysed. We further compared the CD16 bioactivity in sera of healthy individuals with patients with RA, PsA and SLE. The high sIC bioactivity in the serum of patients with SLE was in line with the results from our previous study,¹⁷ and thus confirmed this finding in an independent cohort. We also found a significantly higher sIC bioactivity in RA compared with HCs and PsA, making sIC bioactivity a viable option for differential diagnosis. However, while novel in its discovery, the degree of interpatient variance in circulating sIC bioactivity speaks against sIC quantification as a general RA biomarker. We were also unable to link sIC bioactivity to clinical parameters including a retrospective analysis of medication, disease activity, CRP levels, age and gender. The only meaningful clinical correlation was between CD16 bioactivity in synovial fluid and RF or anti-CCP serum titres, in line with IgG immune complex formation. Future longitudinal measurements will likely reveal possible therapeutic effects on serum CD16 bioactivity. In a patient with systemic sclerosis, we have recently described the disappearance of serum CD16 bioactivity during a combination immunosuppression therapy including CD19.CAR-T cells.^{38 39}

There are limitations intrinsic to this study type, originating from its retrospective character and cryopreservation of serum. Cryopreservation may impact the integrity of immune complexes. Repeated freezing-thawing cycles were avoided to limit the procedural damage of sICs. The focus on CD16 was hypothesis-driven and prevents general statements regarding other FcγRs.

Notably, several healthy donors also showed a low level of circulating sICs or CD16 bioactivity in the serum. This indicates that sICs per se are not exclusively associated with clinically apparent disease, in contrast, it is rather likely that sICs may play a yet ill-defined role in physiological immune responses such as infections.³⁴ The latter does not per se contradict the hypothesis that high levels and chronic persistence of bioactive circulating sICs in RA and especially SLE contribute to autoimmunity,

but raises the obvious question about the causes of their occurrence and its consequences. *Nota bene*, the reporter assay applied in this study only provides information on the principle binding to, and activation of CD16. The CD16 bioactivity measured in the reporter assay does not directly imply the capacity to activate human immune cells *in vivo* bearing this receptor. As described in the introduction, we are not aware of studies that reliably proved the direct activation of CD16-bearing cells by native sICs. Given the large amounts of studies dealing with autoantibodies and also with sICs in the past, it is likely that attempts to measure direct effects on cells were not successful.

Risk-associated CD16 single nucleotide polymorphisms suggest a pathophysiological role of this FcγR in RA. With CD16 bioactivity of sICs in RA being shown here, previous reports of functional relationships between CD16 activation and pathophysiological hallmarks are substantiated. For example, recent insights on the effects of CD16 on osteoclastogenesis and bone loss which had been investigated using heat aggregation-induced sICs²⁶ now seem to be more likely to happen *in vivo*. This supports the hypothesis that sICs regulate bone homeostasis.⁴⁰ In this regard, sICs with CD16 bioactivity are an important missing piece to the puzzle in understanding CD16 biology in RA.

CD16-expressing cells in RA joints include natural killer (NK) cells and monocytes/macrophages. How and whether NK cells contribute to arthritis is not clear. Interestingly, these cells have also been associated with erosiveness⁴¹ and may facilitate bone loss by triggering osteoclastogenesis.⁴² NK cells may act pro-inflammatory by secreting cytokines such as interferon-γ.^{35 41} Synovial monocytes have been recently shown to upregulate genes involved in Fc-receptor signalling in active RA.⁴³ Single cell analyses with ultra-high resolution will help to more precisely characterise CD16-positive synovial cells and their functions in health and disease.⁴⁴ We suggest to add information on sICs for clinical classification and stratification, in order to incorporate this recently neglected but highly important dimension of autoimmunity.^{45 46} Our model may also be helpful in trials targeting immune complex-mediated immune responses.^{47 48}

Aside from the unclear pathogenicity of circulating immune complexes, further important questions regarding sICs cannot be answered by the reporter assay and remain unsolved: What is their size? Are they homogenous or heterogeneous? What are their constituents? In RA, the presence of citrullinated peptides has been suggested, complement is involved and mass spectrometry indicated abundant proteins within enriched proteins aggregated with IgG.^{21 49 50} Also RNA may be incorporated in immune complexes, at least in connective tissue diseases.⁵¹ However, studies on the biochemical characterisation of immune complexes with proven bioactivity at Fc receptors are still pending and will be the next step towards a better understanding of autoimmunity in RA.

CONCLUSION

RA is characterised by the presence of circulating and local sICs that can engage and activate CD16.

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