








Detection and functional resolution of soluble immune complexes by an Fc γ R reporter cell panel

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Abstract

Fc-gamma receptor (Fc γ R) activation by soluble IgG immune complexes (sICs) represents a major mechanism of inflammation in certain autoimmune diseases such as systemic lupus erythematosus (SLE). A robust and scalable test system allowing for the detection and quantification of sIC bioactivity is missing. We developed a comprehensive reporter cell panel detecting activation of Fc γ Rs. The reporter cell lines were integrated into an assay that enables the quantification of sIC reactivity via ELISA or a faster detection using flow cytometry. This identified Fc γ RIIA(H) and Fc γ RIIIA as the most sIC-sensitive Fc γ Rs in our test system. Reaching a detection limit in the very low nanomolar range, the assay proved also to be sensitive to sIC stoichiometry and size reproducing for the first time a complete Heidelberger-Kendall curve in terms of immune receptor activation. Analyzing sera from SLE patients and mouse models of lupus and arthritis proved that sIC-dependent Fc γ R activation has predictive capabilities regarding severity of SLE disease. The assay provides a sensitive and scalable tool to evaluate the size, amount, and bioactivity of sICs in all settings.

Keywords immune complexes; Fc-gamma receptors; Fc γ R activation; SLE

Subject Categories Immunology; Methods & Resources

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Introduction

Immunoglobulin G (IgG) is the dominant immunoglobulin isotype in chronic infections and in various antibody-mediated autoimmune diseases. The multi-faceted effects of the IgG molecule rely both on the F(ab) regions, which recognize a specific antigenic determinant to form immune complexes (ICs), and the constant Fc region (Fc γ),

which is detected by effector molecules like the Fc γ receptors (Fc γ Rs) found on most cells of the immune system or C1q of the complement system. When IgG binds to its antigen ICs are formed, which, depending on the respective antigen, are either matrix/cell-bound or soluble (sICs). The composition of sICs is dependent on the number of epitopes recognized by IgG on a single antigen molecule and the ability of the antigen to form multimers. Fc γ -Fc γ R binding is necessary but not sufficient to activate Fc γ Rs since physical receptor cross-linking is required for receptor triggering (Duchemin *et al.*, 1994; Luo *et al.*, 2010; Patel *et al.*, 2019). IgG opsonized infected cells or microorganisms are readily able to cross-link Fc γ Rs (Bruhns *et al.*, 2009; Lux *et al.*, 2013). This initiates various signaling pathways (Greenberg *et al.*, 1994; Kiefer *et al.*, 1998; Luo *et al.*, 2010), which in turn regulate immune cell effector functions (Nimmerjahn & Ravetch, 2010; Bournazos *et al.*, 2017). It is also suggested that sICs can dynamically tune Fc γ R triggering, implying that changes in sIC size directly impact strength and duration of Fc γ R responses (Lux *et al.*, 2013). However, the molecular requirements are largely unknown and a translation to bioactivity of the paradigmatic Heidelberger-Kendall precipitation curve, describing that sIC size depends on the antigen:antibody ratio, is so far missing (Heidelberger & Kendall, 1929, 1935).

Among all type I Fc γ Rs, Fc γ RIIB (CD32B) is the only inhibitory receptor, signaling via immunoreceptor tyrosine-based inhibitory motifs, while the activating receptors are associated with immunoreceptor tyrosine-based activation motifs. Another exception is Fc γ RIIB (CD16B), which is glycosylphosphatidylinositol-anchored and lacks a signaling motif (Nimmerjahn & Ravetch, 2006, 2008; Bruhns, 2012; Bruhns & Jonsson, 2015). Still, Fc γ RIIB is widely accepted to be a neutrophil activating receptor, e.g. by cooperating with other Fc γ Rs such as Fc γ RIIA (Vossebeld *et al.*, 1997). Fc γ RI (CD64) is the only high affinity Fc γ R binding also to monomeric IgG, while all other Fc γ Rs only efficiently bind to complexed, i.e. antigen-bound IgG (Bruhns, 2012; Bruhns & Jonsson, 2015; Lu *et al.*, 2018). Activation of Fc γ Rs leads to a variety of cellular effector functions elicited by several immune cells such as natural killer (NK) cells via Fc γ RIIC/Fc γ RIIIA, monocyte-derived cells via Fc γ RI/Fc γ RIIB/Fc γ RIIIA, granulocytes via Fc γ RI/Fc γ RIIA/Fc γ RIIB, platelets

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via Fc γ RIIA and B cells via Fc γ RIIB. Consequently, Fc γ Rs connect and regulate both the innate and adaptive branches of the immune system. Various factors have been shown to influence IC-dependent Fc γ R activation profiles, including Fc γ R-Fc γ binding affinity and avidity (Koenderman, 2019), IgG subclass, glycosylation patterns and genetic polymorphism (Bruhns *et al*, 2009; Pincetic *et al*, 2014; Vidarsson *et al*, 2014; Plomp *et al*, 2017), stoichiometry of antigen-antibody-ratio (Berger *et al*, 1996; Pierson *et al*, 2007; Lux *et al*, 2013), and Fc γ R clustering patterns (Patel *et al*, 2019). Specifically, Asn297-linked glycosylation patterns of the IgG Fc domain initiate either pro- or anti-inflammatory effector pathways by tuning the binding affinity to activating versus inhibitory Fc γ Rs, respectively (Bohm *et al*, 2014). However, despite being explored in proof-of-concept studies, the functional consequences of these ligand features on a given Fc γ R are still not fully understood. Therefore, there is an obvious need for an assay platform allowing for the systematic assessment of IC-mediated Fc γ R activation.

Soluble IgG immune complexes and immobilized ICs represent unequal and discrete stimuli for the immune system (Fossati *et al*, 2002; Granger *et al*, 2019). Soluble circulating ICs are commonly associated with certain chronic viral or bacterial infections (Wang & Ravetch, 2015; Yamada *et al*, 2015) and autoimmune diseases, such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA; Koffler *et al*, 1971; Zubler *et al*, 1976; Antes *et al*, 1991). When deposited and accumulating in tissues, sICs can cause local damage due to inflammatory responses, classified as type III hypersensitivity (Rajan, 2003). Compared with immobilized local ICs, which recruit immune cells causing tissue damage (Mulligan *et al*, 1991; Mayadas *et al*, 2009; Ward *et al*, 2016), sIC related disorders are characterized by systemic inflammation, which is reflected by immune cell exhaustion and senescence (Tahir *et al*, 2015; Chauhan, 2017; Bano *et al*, 2019). In order to resolve sIC-dependent activation of Fc γ Rs in greater detail we developed a scalable reporter system suited for two high throughput readouts, capable of quantifying and distinguishing the activation of single Fc γ Rs. As the assay is also sensitive to stoichiometry and sIC size, we were able to translate the Heidelberger-Kendall precipitation curve to Fc γ R bioactivity. Compared to currently available ELISA assays detecting sICs by their affinity to C1q-CIC (circulating ICs) or C3d, the assay system presented below is strictly specific for IgG ICs and integrates sICs of all sizes into single Fc γ receptor bioactivity. Applying reporter cell lines enables very high sensitivity in the low nanomolar range, as signals are biologically amplified compared to biochemical binding based read-outs. Finally, we applied the assay to a clinical setting, measuring sICs in sera from SLE patients. A reporter cell panel

expressing murine Fc γ Rs revealed the detection of sICs in the serum of autoimmune-prone diseased mice in preclinical models of lupus and arthritis. Prospectively, this methodology could be instrumental as an experimental and clinical toolbox to unveil sIC-mediated Fc γ R activation in various autoimmune or infectious diseases.

Results

Experimental assay setup

The assay used in this study was adapted from a previously described cell-based Fc γ R activation test system designed to measure receptor activation in response to opsonized virus infected cells (Corrales-Aguilar *et al*, 2013; Kolb *et al*, 2021) and therapeutic Fc-fusion proteins (Lagasse *et al*, 2019). We refined the assay to enable selective detection of sICs and expanded the reporter cell line-up (Fc γ RI: Acc# LT744984; Fc γ RIIA (131R): Acc# M28697; Fc γ RIIA (131H): Acc# XP_011507593; Fc γ RIIB/C: Acc# LT737639; Fc γ RIIIA (176V): Acc# LT737365; Fc γ RIIIB(176V): Acc# O75015). Ectodomains of Fc γ RIIB and Fc γ RIIC are identical. Second generation reporter cells were generated to improve stable expression of chimeric Fc γ Rs compared to the transfectants used in the original assay (Corrales-Aguilar *et al*, 2013). To this end, mouse BW5147 cells were transduced as described previously via lentiviral transduction (Halenius *et al*, 2011; Corrales-Aguilar *et al*, 2013; Van den Hoecke *et al*, 2017). Human Fc γ R expression on transduced cells after puromycin selection and two consecutive cell sorting steps was assessed by flow cytometry (Fig 1A). Fc γ R activation is measured by surface CD69 expression after 4 h of incubation using high throughput flow cytometry or by quantification of IL-2 secretion after 16 h of incubation using ELISA. Suspension of IgG or sICs in the liquid phase is enforced by pre-incubation of a 96 well ELISA microtiter plate with PBS/FCS blocking buffer (Fig 1B). To this end, we compared graded concentrations of FCS in the blocking reagent and measured the threshold at which IgG was no longer adsorbed to the plate and stayed abundantly in solution. FCS supplementation to 1% (v/v) or higher is sufficient to keep IgG antibodies in solution. We then set out to test if immobilized IgG can be used as an operational surrogate for IgG-opsonized cells or immobilized ICs with regard to Fc γ R activation as suggested previously (Tanaka *et al*, 2009). We found no qualitative difference in Fc γ R activation between immobilized Rtx, immobilized ICs (Rtx + rec. CD20) or Rtx-opsonized 293T-CD20 cells (Fig 1C). In contrast, sICs formed by monomeric CD20 antigen (aa 141–188) and Rtx failed to activate Fc γ Rs even at very

Figure 1. Establishment of a cell-based reporter assay measuring Fc γ R activation in response to sICs.

- BW5147 reporter cells stably expressing human Fc γ R- ζ chain chimeras or BW5147 parental cells (grey/dashed) were stained with Fc γ R specific conjugated mAbs as indicated and measured for surface expression of Fc γ Rs via flow cytometry. Bar graphs show means of three independent experiments performed in technical duplicates. Error bars = SD.
- FCS coating of an ELISA microtiter plate allows for suspension of subsequently added IgG. Plate bound IgG was quantified via ELISA. Bar graph shows means of one experiment performed in technical triplicates. Error bars = SD.
- Immobilized IC, immobilized IgG and IgG opsonized cells represent qualitatively similar ligands for Fc γ Rs. Response curves of human Fc γ Rs activated by opsonized cells (293T cells stably expressing CD20 + Rituximab [Rtx]), immobilized IC (rec. soluble CD20 + Rtx) and immobilized IgG (Rtx). sICs formed using monovalent antigen (rec. soluble CD20 + Rtx) do not activate human Fc γ Rs. X-Axis shows sample concentration determined by antibody molarity. Y-Axis shows Fc γ R activation determined by reporter cell mouse IL-2 production (OD 450 nm). Two independent experiments performed in technical duplicates. Error bars = SD.
- Schematic of used assay setups. BW5147 reporter cells expressing chimeric human Fc γ R receptors express endogenous CD69 or secrete mouse IL-2 in response to Fc γ R activation by clustered IgG. sICs are generated using mAbs and multivalent antigens. sIC suspension requires pre-blocking of an ELISA plate using PBS supplemented with 10% FCS (FCS coat, grey-dashed).

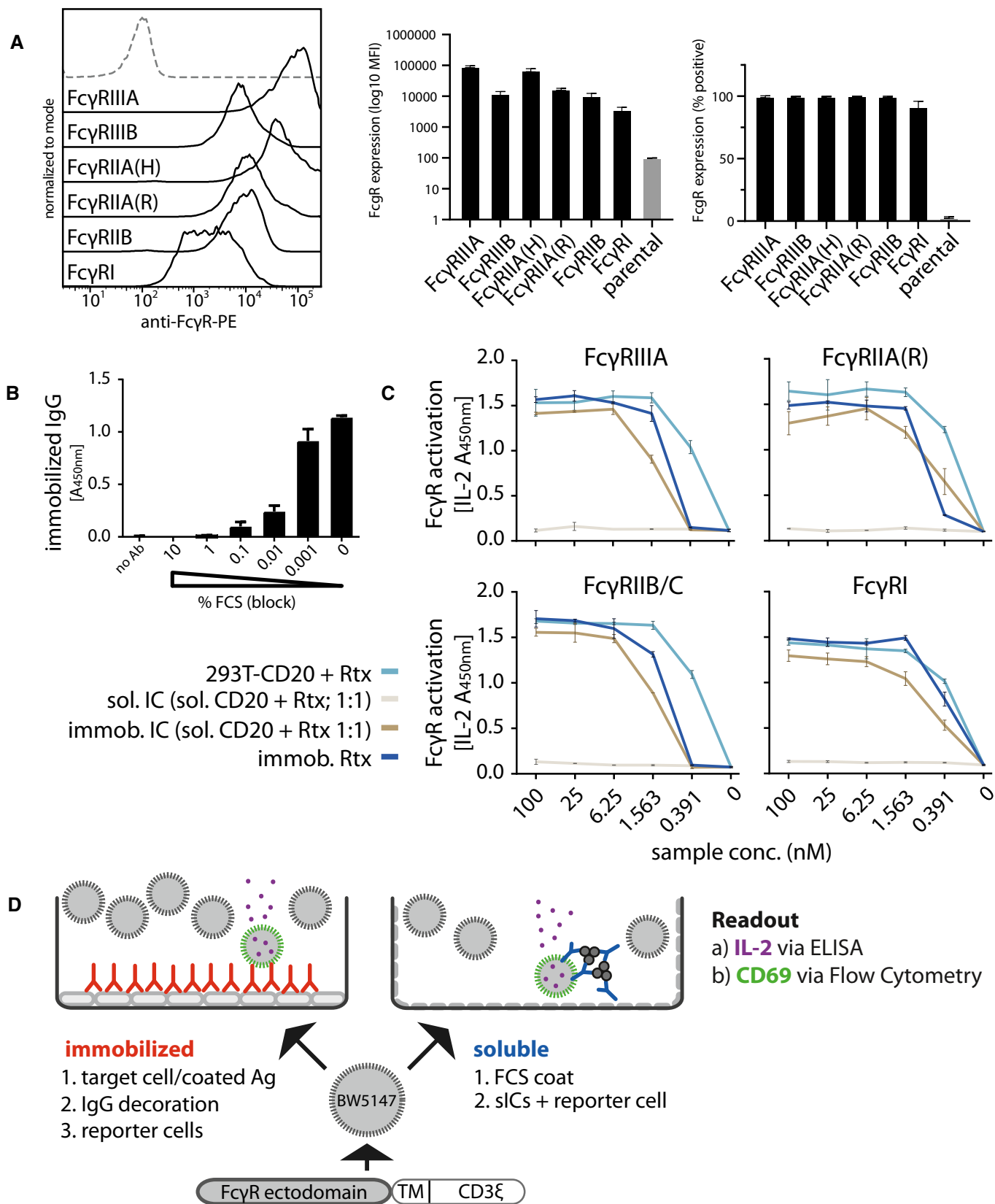


Figure 1.

high ligand concentrations. We concluded that Fc γ R-crosslinking by sICs is only achieved by multivalent antigens but not dimeric ICs. Of particular note, to reliably and accurately differentiate between strictly soluble and immobilized including aggregated triggers using this assay, reagents for the generation of synthetic ICs needed to be of therapy-grade purity. The assay setup is depicted in Fig 1D.

Detection of human Fc γ R activation by multimeric sICs

Next, we generated synthetic sICs from recombinant ultrapure molecules to evaluate the assay. We aimed to avoid the use non-human molecules, misfolded IgG aggregates, or IgG-IgG complexes to generate a most native and defined ligand. To date, there are still few commercially available human IgG-antigen pairs that meet both the above mentioned high grade purity requirements while also consisting of at least two antigen monomers. In order to meet these stipulations we focused on three pairs of multivalent antigens and their respective mAbs that were available in required amounts enabling large-scale titration experiments; trimeric TNF- α :IgG1 infliximab (TNF- α :Ifx), dimeric rhVEGFA: IgG1 bevacizumab (VEGFA/Bvz) and dimeric rhIL-5: IgG1 mepolizumab (IL-5/Mpz). As lymphocytes express TNF- α -receptors I and II while not expressing receptors for IL-5 or VEGFA, we tested whether the mouse lymphocyte derived BW5147 thymoma reporter cell line is sensitive to high concentrations of rhTNF- α . Toxicity testing revealed that even high concentrations of up to 76.75 nM TNF- α did not affect viability of reporter cells (Fig EV1A). Next, we measured the dose-dependent activation of human Fc γ Rs comparing immobilized IgG to sICs (TNF- α :Ifx) using the full Fc γ R reporter cell panel (Fig 2). Soluble antigen or mAb alone served as negative controls showing no background activation even at high concentrations. Immobilized rituximab (Rtx, human IgG1) and immobilized Fc γ R-specific mouse mAbs served as positive controls for inter-experimental reference. All Fc γ Rs were activated by immobilized IgG. Only Fc γ RI failed to respond when reporter cells were incubated with sICs. Both the IL-2 ELISA as well as the CD69 expression read-out gave comparable results. Further, using an IL-2 standard, we were able to quantify Fc γ R activation (right y-axis). This revealed that the reporter cell lines differ regarding reactivity, which did not correlate with receptor expression. Although the low signals for Fc γ RI might be linked to receptor expression, responsiveness was markedly lower compared to other reporter cell lines (Fig 1A). Attempts to increase and equalize receptor expression by repeated cell sorting steps failed, indicating that individual receptors only tolerate limited molecule densities on the reporter cell surfaces. To control for the specificity of the observed reporter cell activation, we also tested sICs (TNF- α :Ifx) generated from F(ab')₂ fragments instead of full-length IgG. This showed that Fc γ R activation by sICs is Fc γ dependent

(Fig EV1B). Finally, we challenged sIC mediated reporter cell activation with increasing amounts of IVIg. Even when reaching a 50-fold excess of IVIg over Ifx, no impairment of Fc γ RIII activation confirming a large tolerance for high amounts of non-complexed monomeric IgG (Fig EV1C), which is a potential concern when testing patient derived samples. This is in line with Fc γ Rs showing a higher affinity for complexed IgG over monomeric IgG (Bruhns *et al*, 2009).

Evaluation of human Fc γ R activation by multimeric sICs

The assay proved to be sensitive to sICs in the nanomolar range. Regarding immobilized IgG, the detection limit was between 1 and 3 nM. sICs were detected with the following limits regarding the IL-2 readout: Fc γ RI—no detection; Fc γ RIIA(R)—3 nM; Fc γ RIIA(H)—0.2 nM; Fc γ RIIB/C—3 nM; Fc γ RIIIA—0.2 nM; Fc γ RIIIB—25 nM. We observed that sICs and immobilized ICs induce largely different signal strength in individual reporter cell lines. Fc γ RI and Fc γ RIIIB were more efficiently activated by immobilized IgG compared to sICs. Conversely, Fc γ RIIA(H) and Fc γ RIIIA were more efficiently activated by sICs. Fc γ RIIB/C showed discrepant results when comparing the IL-2 read-out (16 h) with the CD69 read-out (4 h). Here, it seems that a longer activation leads to a stronger signal on immobilized IgG, while shorter activation slightly favors sIC reactivity. Fc γ RII(R) looked similar to Fc γ RIIB/C with a slightly higher response to sICs at low stimulant concentrations. Nevertheless, the response to immobilized IgG was higher for both read-outs at higher concentrations. Fc γ RIIA(H) and Fc γ RIIIA proved to be the most sensitive towards sICs stimulation. Notably, the reported superior interactivity of sICs with Fc γ RII (H) over Fc γ RII (R) (Shashidharamurthy *et al*, 2009) was not only confirmed using our assay but we also show that this difference is limited to sIC reactivity and is not seen in immobilized IC reactivity (Fig 2). Additionally, we measured the response of select reporter cell lines towards sICs of different composition (VEGFA/Bvz and IL-5/Mpz). As these sICs incorporate dimeric antigens, we tested if reporter responses were still comparable (Fig EV2). We observed that responses to sICs were generally lower for Fc γ RIIA(R) but comparable for Fc γ RI, Fc γ RIIB/C and Fc γ RIIIA. Of note, Fc γ RI showed slight reactivity towards VEGFA/Bvz sICs. Based on the universal transmembrane and cytosolic part of the Fc γ R chimeras in our assay we concluded that Fc γ R ectodomains are intrinsically able to differentiate between different conformations of sICs and immobilized monomeric IgG ligands. To validate the data generated by the reporter assay, we determined Fc γ RIIIA activation using primary human NK cells isolated from PBMCs of three healthy donors. We chose NK cells as they mostly express only one type of Fc γ R similar to the reporter system and used IL-5/Mpz sICs as NK cells do not respond to IL-5. Measuring a

Figure 2. Fc γ Rs are activated by sICs formed from multivalent antigens.

Ultra-pure antigen (Ag, TNF- α) mixed with therapy-grade mAb (infliximab, Ifx) was used to generate sICs. X-Axis: concentrations of stimulant expressed as molarity of either mAb or Ag monomer and IC (expressed as mAb molarity) at a mAb:Ag ratio of 1:2. Soluble antigen or soluble antibody alone served as negative controls and were not sufficient to activate human Fc γ Rs. Immobilized IgG (Rtx) or immobilized Fc γ R-specific mAbs served as positive controls. Two independent experiments performed in technical duplicates. Error bars = SD. Error bars smaller than symbols are not shown. Left panel: IL-2 quantification 16 h after reporter cell activation. Background (blank) was subtracted (dashed line). IL-2 was measured via anti-IL-2 ELISA (A_{450nm}) and IL-2 concentrations were calculated from an IL-2 standard. Right panel: Reporter cell CD69 expression 4 h post trigger was measured using flow cytometry. Mean fluorescence intensity (MFI) were normalized to untreated cells (ctrl.) and are presented as fold-change increase.

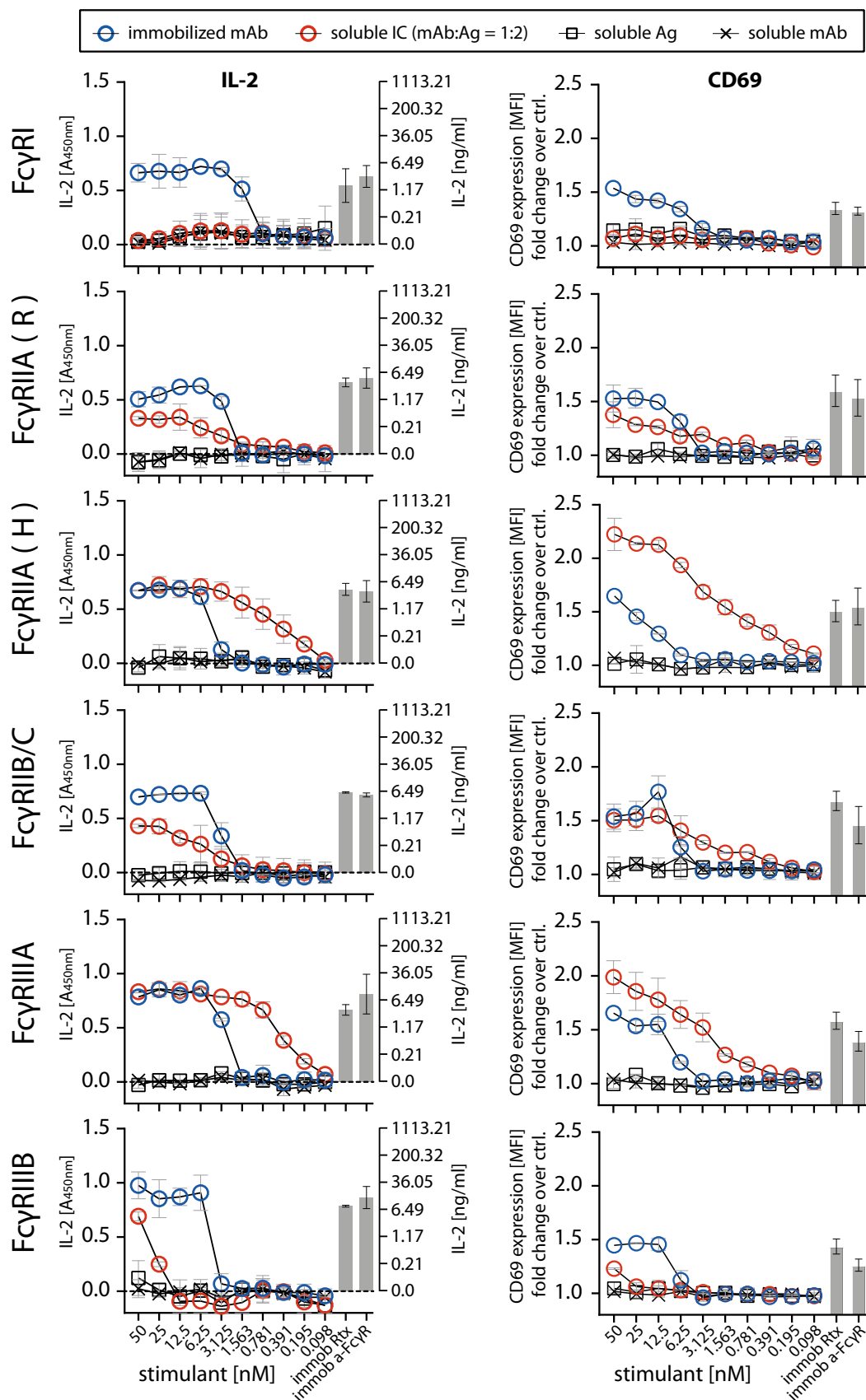


Figure 2.

panel of activation markers and cytokine responses by flow cytometry, we observed a differential activation pattern depending on ICs being soluble or immobilized at equal molarity (Fig 3A). While MIP1- β responses were comparable between the two triggers, degranulation (CD107a) and TNF- α responses showed a trend towards lower activation by sICs compared to immobilized IgG (Mpz). Strikingly, IFN γ responses were significantly weaker when NK cells were incubated with sICs compared to immobilized IgG. Next, in order to confirm this to be due to specific activation of Fc γ RIIIA, we changed the sIC setup by generating reverse-orientation sICs consisting of human Fc γ R-specific mouse mAbs and goat-anti-mouse IgG F(ab')₂ fragments (Fig EV3A). NK cell activation by reverse sICs was compared to NK cell activation by immobilized Fc γ R specific mAbs. This confirmed our previous observations. As in roughly 10% of the population NK cells express Fc γ RIIC (Metes *et al*, 1998; Breunis *et al*, 2008; Lisi *et al*, 2011; Anania *et al*, 2019), we also tested reverse sIC activation using an Fc γ RII specific mAb. As we did not observe an Fc γ RII-mediated response, we conclude that Fc γ RIIC expression did not play a role in our experiments (Fig EV3B). Importantly, these experiments validate that all our experimentally synthesized sICs readily activate primary NK cells and induce immunological effector functions.

Next, primary neutrophils isolated from whole blood samples of four individual donors were analyzed, measuring upregulation of CD11B, CD66B, and shedding of L-selectin as markers of IC mediated adhesion and activation (Ilton *et al*, 1999; Lard *et al*, 1999; Zarbock & Ley, 2009; Khawaja *et al*, 2019; Fig 3B). Again, immobilized IgG and sICs activated primary neutrophils with different efficiency. While both, sICs and immobilized IgG, strongly induced the shedding of L-selectin, the upregulation of CD11B and CD66B showed a tendency towards lower activation by sICs compared to immobilized IgG. As neutrophils express both Fc γ RIIA and Fc γ RIIB, we also individually activated these receptors on neutrophils using immobilized Fc γ R-specific mAbs. This revealed that neutrophil activation by Fc γ Rs is mostly driven by Fc γ RIIB. In light of our previous tests (Fig 2), this explains the reduced activation by sICs. Taken together, we conclude that primary cells differentiate between opsonized targets and sICs via inherent features of individual Fc γ Rs as well as by the co-expression of Fc γ Rs with different sensitivity towards sICs.

Measurement of Fc γ R activation in response to the molecular size of sICs

We observed that the dimeric CD20:Rtx molecule complex completely failed to trigger Fc γ R activation (Fig 1C) while potentially larger sICs, based on multimeric antigens, showed an efficient dose-dependent Fc γ R activation (Figs 2 and EV2). In order to determine whether Fc γ R signaling responds to changes in sIC size, we cross-titrated amounts of antibody (mAb, infliximab, Ifx) and antigen (Ag, rhTNF- α). Specifically, the reporter cells were incubated with sICs of varying mAb:Ag ratio by fixing one parameter and titrating the other. According to the Heidelberger-Kendall precipitation curve (Heidelberger & Kendall, 1929), sIC size depends on the mAb:Ag ratio. sICs of varying sizes result from an excess of either antigen or antibody, leading to the formation of smaller complexes compared to the large molecular complexes formed at around equal molarity. Presumed changes in sIC size were quantified using asymmetrical flow-field flow fractionation (AF4; Fig 4A and Table 1). AF4 analysis revealed

a sIC-mean molecular weight of ~2,130 kDa at a 1:3 ratio (Ifx/TNF- α) with sICs getting smaller with increasing excess of either antigen or antibody, recapitulating a Heidelberger-Kendall-like curve. Incubation of the Fc γ R reporter cells with sICs of varying size indeed shows that the assay is highly sensitive to changes in sIC size (Fig 4B). Accordingly, Fc γ Rs showed the strongest responses at mAb:Ag ratios of ~1:3. Next, we validated the accuracy of our reporter cell data by subjecting primary human NK cells to the same variation of mAb:Ag stoichiometry. NK cells from three individual donors were measured for MIP1- β upregulation in response to synthetic sICs of varying size and composition (Fig 4C). Indeed, primary NK cells equally responded to sIC size at the same nanomolar range of stimulating ligand, confirming that the reporter system accurately measures immune cell responses to sICs. Convincingly, NK cell responses to sICs generated from trimeric antigen (TNF α) peaked at a different mAb:Ag ratio compared to NK cell responses to sICs generated from dimeric antigens (IL-5 and VEGFA). TNF- α and VEGFA contribute to the activation of resting NK cells, thus leading to higher MIP1- β positivity when NK cells are incubated in the presence of excess antigen. As NK cells do not express IL-5 receptor, this effect is not observed in the presence of excess IL-5. The data reveal a direct correlation between sIC dimension and effector responses. Conversely, when changing antibody concentrations using fixed amounts of antigen, a consistent reduction of NK cell activation is observed in the presence of excess IgG for all three mAb:Ag pairs.

Quantification of sIC bioactivity in sera of SLE patients

In order to apply the assay to a clinically relevant setting associated with the occurrence of sICs, we measured circulating sICs present in the serum of SLE patients with varying disease activity. Sera from 4 healthy donors and 25 SLE patients were investigated for Fc γ RIIIA and Fc γ RIIB/C activation to compare an activating and an inhibitory receptor. Reporter cells readily secreted mIL-2 in response to patient sera in a dose-dependent manner (Fig 5A), which was not the case when sera from healthy controls were tested. We confirm that Fc γ RIIIA and Fc γ RIIB/C activation depends on the presence of serum sICs by comparing the bioactivity of patient serum before and after polyethylene glycol (PEG) precipitation which is known to deplete sICs (Lux *et al*, 2013; Fig 5B). Next, we calculated the area under the curve (AUC) values for all 25 SLE patient titrations and normalized them to the AUC values measured for healthy individuals. The resulting index values were then correlated with patient SLE Disease Activity Index (SLEDAI-2K; Gladman *et al*, 2002; Fig 5C) or conventional biomarkers of SLE disease activity, being anti-dsDNA titers (α -dsDNA) and concentrations of the complement cleavage product C3d (Fig 5D). Fc γ RIIIA activation index values showed significant correlation with all conventional index values (SLEDAI: $P = 0.0109$; α -dsDNA: $P = 0.0465$; C3D: $P = 0.0052$). Fc γ RIIB/C activation showed no significant correlation, respectively. We assume these interrelations may be due to the influence of IgG sialylation found to be reduced in active SLE (Vuckovic *et al*, 2015). Generally, de-sialylation of IgG leads to stronger binding by the activating receptors Fc γ RI, Fc γ RIIA, and Fc γ RIII while it reduces the binding affinity of the inhibitory Fc γ RIIB (Kaneko *et al*, 2006). In practice, our assay allows the detection and quantification of clinically relevant sICs in sera from SLE patients as shown here or in synovial fluid of RA patients (Zhao *et al*, 2021).

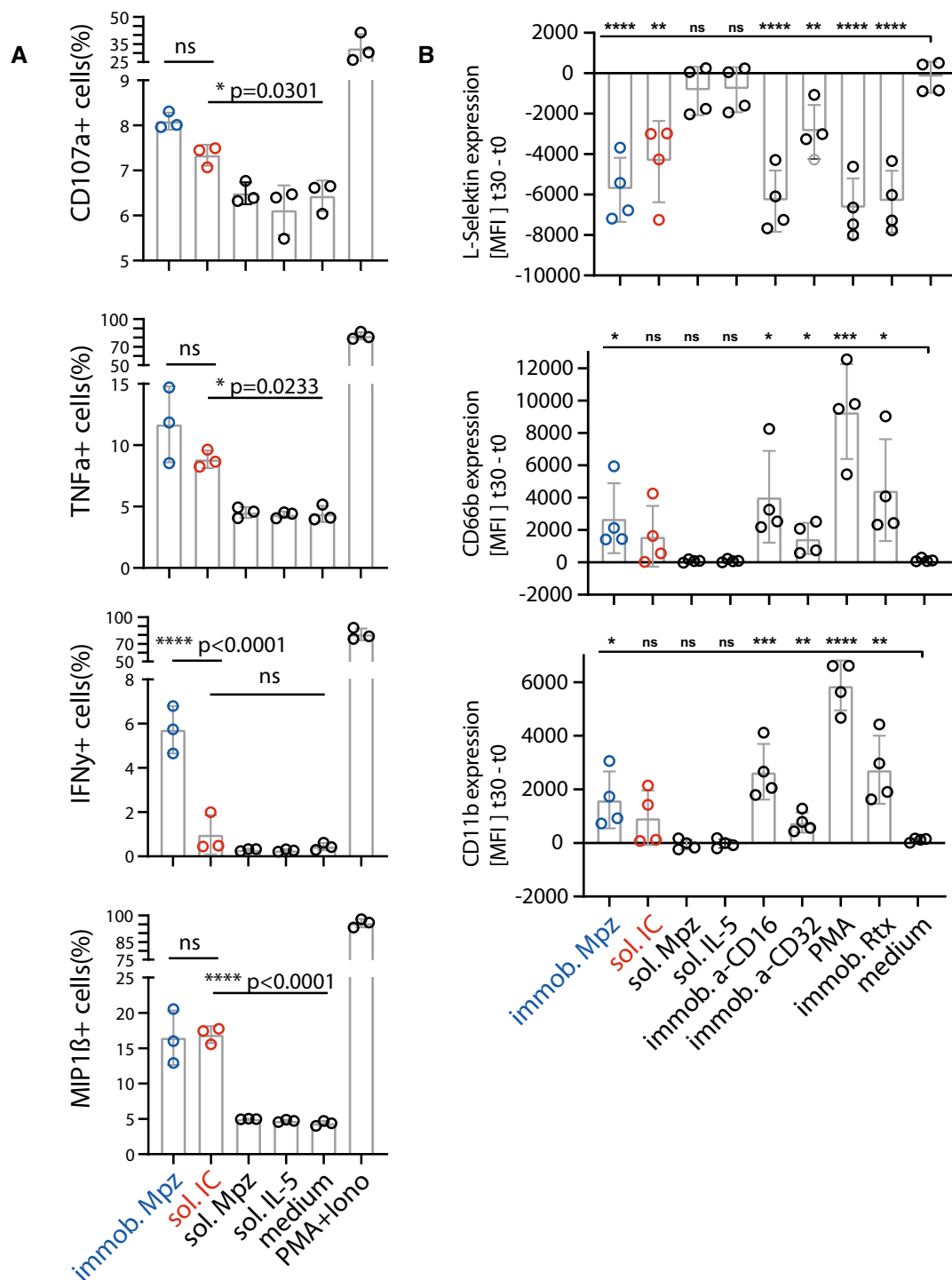


Figure 3. The FcγR-dependent activation pattern of primary NK cells or primary neutrophils depends on IC solubility.

A Negatively selected primary NK cells purified from PBMCs of three healthy donors were tested for activation markers using flow cytometry. NK cells were incubated with immobilized IgG (mepolizumab, Mpz), soluble IC (Mpz:IL-5 = 1:1), soluble Mpz or soluble IL-5 (all at 200 nM, 10^6 cells). Incubation with PMA and Ionomycin (Iono) served as a positive control. Incubation with medium alone served as a negative control. Each dot represents one donor measured in technical duplicates. Error bars = SD. One-way ANOVA (Tukey); * $P < 0.05$, **** $P < 0.0001$.

B Negatively selected primary neutrophils purified from whole blood of four healthy donors were tested for adhesion and activation markers using flow cytometry. Neutrophils were incubated with immobilized IgG (Mpz), soluble IC (Mpz:IL-5 = 1:1), soluble Mpz or soluble IL-5 (all at 200 nM, 2×10^5 cells). Incubation with PMA or immobilized rituximab served as positive controls. Incubation with medium served as a negative control. Immobilized FcγRII and FcγRIII specific mAbs served as functional controls. MFI values at $t = 30$ min of incubation are presented as increase over $t = 0$ min. Each dot represents one donor measured in technical duplicates. Error bars = SD. Two-way ANOVA compared to medium (Dunnett); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

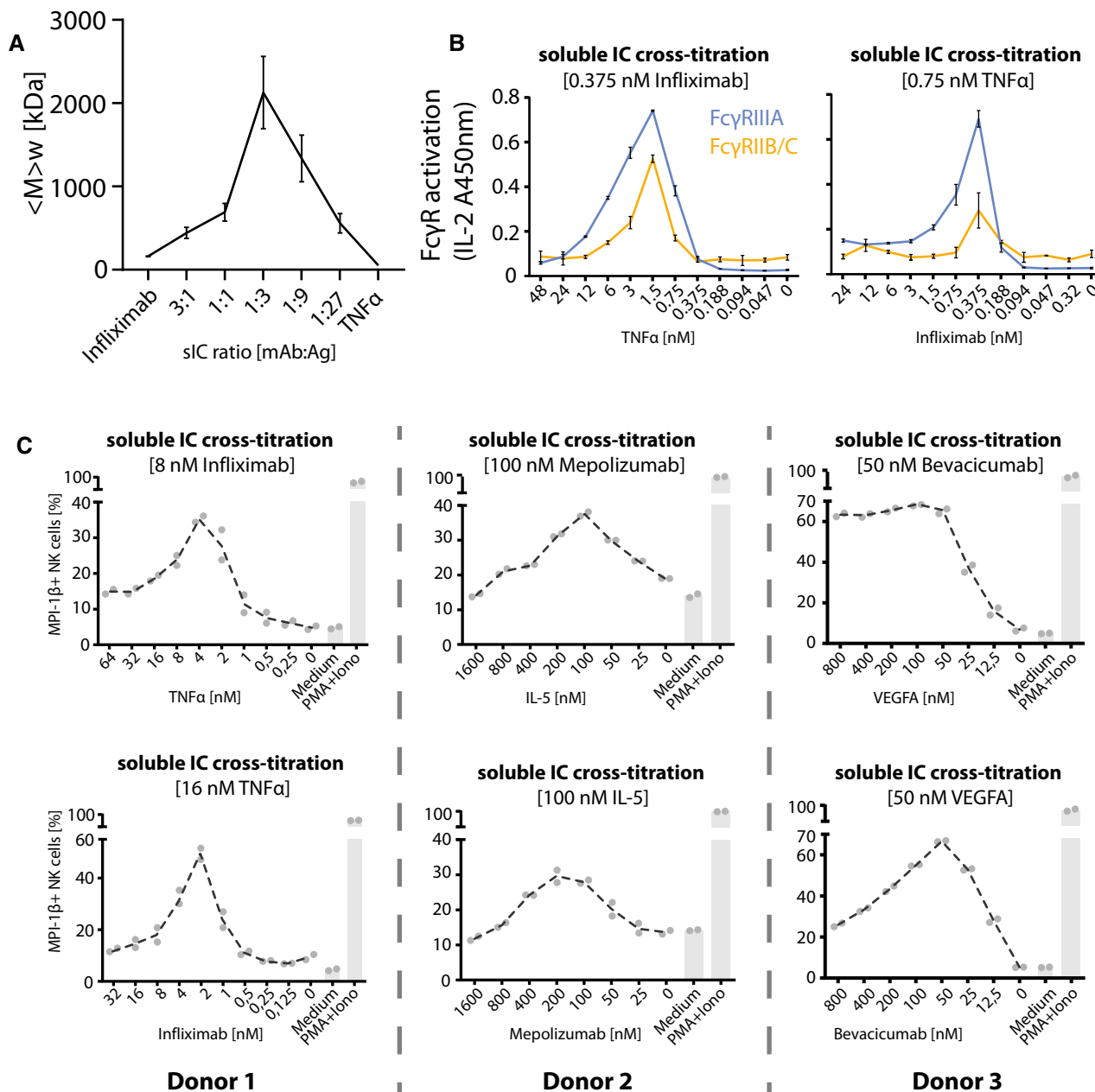


Figure 4. Fc γ RIIB/C and Fc γ R-IIIa respond to sIC size reproducing a Heidelberger-Kendall like precipitation curve.

A Infliximab (mAb) and rhTNF- α (Ag) were mixed at different ratios (17 μ g total protein, calculated from monomer molarity) and analyzed via AF4. sIC size is maximal at a 1:3 ratio of mAb:Ag and reduced when either mAb or Ag are given in excess. $\langle M \rangle_w$ = mass-weighted mean of the molar mass distribution. Three independent experiments. Error bars = SD. Data taken from Table 1.

B sICs of different size were generated by cross-titration according to the AF4 measurement. Reporter cells were incubated with fixed amounts of either mAb (infliximab, left) or Ag (rhTNF- α , right) and titrated amounts of antigen or antibody, respectively. X-Axis shows titration of either antigen or antibody, respectively (TNF- α calculated as monomer). Two independent experiments performed in technical duplicates. Error bars = SD.

C Purified primary NK cells from three different donors were incubated with cross-titrated sICs as in B. NK cells were measured for MIP-1 β expression (% positivity). Incubation with PMA and Ionomycin served as a positive control. Incubation with medium alone served as a negative control. Each dot represents one experimental replicate; the dashed line represents the mean values.

Assay application to *in vivo* mouse models of lupus and arthritis

BW5147 reporter cells stably expressing chimeric mouse as well as rhesus macaque Fc γ Rs have already been generated using the here

described methodology (Kolb *et al*, 2019; Van den Hoecke *et al*, 2017). Next, we aimed to translate the assay to clinically relevant mouse models. Fc γ R reporter cells expressing chimeric mouse Fc γ Rs were incubated with sera from lupus (NZB/WF1; Dubois *et al*,

Table 1. Analysis of the molar mass distribution of ICs from AF4 data.

Sample	Range of assigned molar masses (kDa)			Mass-weighted mean of assigned molar masses (kDa)			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Mean ± SD
Infliximab, IFX	158–182	153–164	159–193	162	156	163	160 ± 4
TNF-alpha	52–55	51–61	52–62	52	52	52	52 ± 0
ICs							
IFX/TNF 3:1	182–1.16 × 10 ³	164–1.11 × 10 ³	193–1.10 × 10 ³	409	398	518	442 ± 66
IFX/TNF 1:1	182–2.06 × 10 ³	164–1.31 × 10 ³	193–1.42 × 10 ³	801	589	681	690 ± 106
IFX/TNF 1:3	182–5.05 × 10 ³	164–6.89 × 10 ³	193–10.8 × 10 ³	1.77 × 10 ³	2.00 × 10 ³	2.61 × 10 ³	2.13 × 10 ³ ± 435
IFX/TNF 1:9	182–5.36 × 10 ³	164–3.38 × 10 ³	193–3.51 × 10 ³	1.66 × 10 ³	1.18 × 10 ³	1.17 × 10 ³	1.34 × 10 ³ ± 279
IFX/TNF 1:27	182–1.68 × 10 ³	164–768	193–1.01 × 10 ³	689	464	521	558 ± 117

For a given elution time, the AF4 profiles provide the concentration (UV) at which a given molar mass (MALS) of a protein is present in the sample. The molar mass distribution of IFX, TNF- α and their immune complexes (sICs) was obtained by plotting the cumulative frequency as a function of molar mass. For a selected range of molar masses, a mass-weighted mean value ($\langle M_w \rangle$) was calculated. All detected molar masses were selected in the case of IFX and TNF- α whereas only molar masses larger than the maximal molar mass found for IFX were assigned to sICs. The table shows the range of assigned molar masses and the calculated $\langle M_w \rangle$ for each AF4 run ($n = 3$).

1966) or arthritis (K/BxN; Kouskoff *et al*, 1996) mice with symptomatic disease. We chose to determine the stimulation of the activating receptors, mFc γ RIII and mFc γ RIV. Incubation with synthetic sICs generated from rhTNF- α and mouse-anti-hTNF- α IgG1 showed both of the reporters to be equally responsive to sICs (Fig 6A). Parental BW5147 cells expressing no Fc γ Rs served as a control. The sera of three mice per group were analyzed and compared to sera from wildtype C57BL/6 mice, which served as a healthy control. C57BL/6 mice were chosen, as K/BxN or NZB/WF1 mice show temporal variability in disease onset and presymptomatic phase. We consistently detected mFc γ R activation by sera from K/BxN or NZB/WF1 but not healthy C57BL/6 mice (Fig 6B). While the mFc γ RIII responses were generally high and similar between K/BxN and NZB/WF1 mice, mFc γ RIV responsiveness tended to be lower and individually more variable. Altogether, the assay enables the reliable detection of sICs in sera of mice with immune-complex mediated diseases making it a promising novel research tool to study the role of sIC formation and Fc γ R activation in preclinical mouse models.

Discussion

In this study, we established, validated, and applied a new assay system that is able to selectively detect soluble multimeric ICs as

discrete ligands of Fc γ Rs. Our system is sensitive to the size, concentration and composition of sICs. The assay is scalable and supports measurement with human and mouse Fc γ Rs. It provides two readouts suitable for high-throughput analysis: fast CD69 surface expression and quantifiable IL-2 secretion.

A novel assay for the quantification of individual Fc γ R activation by experimental and clinical sICs

Our methodology provides a comprehensive system, supporting the assessment of essentially all Fc γ Rs, which presents an advantage over previously developed sIC detection and Fc γ R activation assays (Cheng *et al*, 2014; Tada *et al*, 2014; Szitiner *et al*, 2016; Hsieh *et al*, 2017; Stopforth *et al*, 2018; Aoyama *et al*, 2019). In contrast to currently available commercial assays, detecting sICs by C1q-CIC or C3d ELISA in the micromolar range, our assay measures overall sIC bioactivity in the nanomolar range and has a sole specificity for IgG sICs. The new approach presents with hands on technical advances as it allows for the measurement of small or large amounts of samples by a relatively simple *in vitro* assay with high-throughput potential. Favorably, BW5147 reporter cells are largely inert to human cytokines, which provides a key advantage to measure their responsiveness after contact with human samples. Our pilot study demonstrates that sIC-mediated Fc γ RIII activation correlates with

Figure 5. The reporter assay enables quantification of serum-derived sICs from SLE patients.

Serum derived sIC from SLE patients activate human Fc γ R reporter cells; 25 patients and 4 healthy control individuals were separated into three groups for measurement.

- A Experiments shown for an exemplary group of eight SLE patients and two healthy individuals. Dose-dependent reactivity of Fc γ Rs IIIA and IIB/C was observed only for SLE patient sera and not for sera from healthy individuals. Each titration represents one donor measured in technical duplicates.
- B Activation of Fc γ Rs IIB/C and IIIA by patient serum is mediated by serum derived sICs. Patient serum samples were depleted of sICs by PEG precipitation and the supernatant (SN) was compared to untreated serum regarding Fc γ R activation (left). Shown is one exemplary experiment performed in technical duplicates. Error bars indicate reproducibility as range. IgG concentration in the precipitate (PC), supernatant (SN) or unfractionated serum respectively is shown in the bar graph (right).
- C, D Correlation of Fc γ R activation data from A with (C) patient SLEDAI or (D) conventional SLE disease markers (α -dsDNA levels indicated as IU/ml or C3d concentrations indicated as mg/L). Left panel: Fc γ RIIIa; right panel: Fc γ R IIB/C. Fc γ R activation from a dose-response curve as in A was calculated as area under curve (AUC) for each SLE patient represented by individual dots. AUC from SLE patients are expressed as fold change compared to the healthy control mean ($n = 4$). SLE patients with α -dsDNA levels below 50 IU/ml and C3d values below 6 mg/l were excluded. One-tailed Spearman's. Dotted lines show 95% confidence bands.

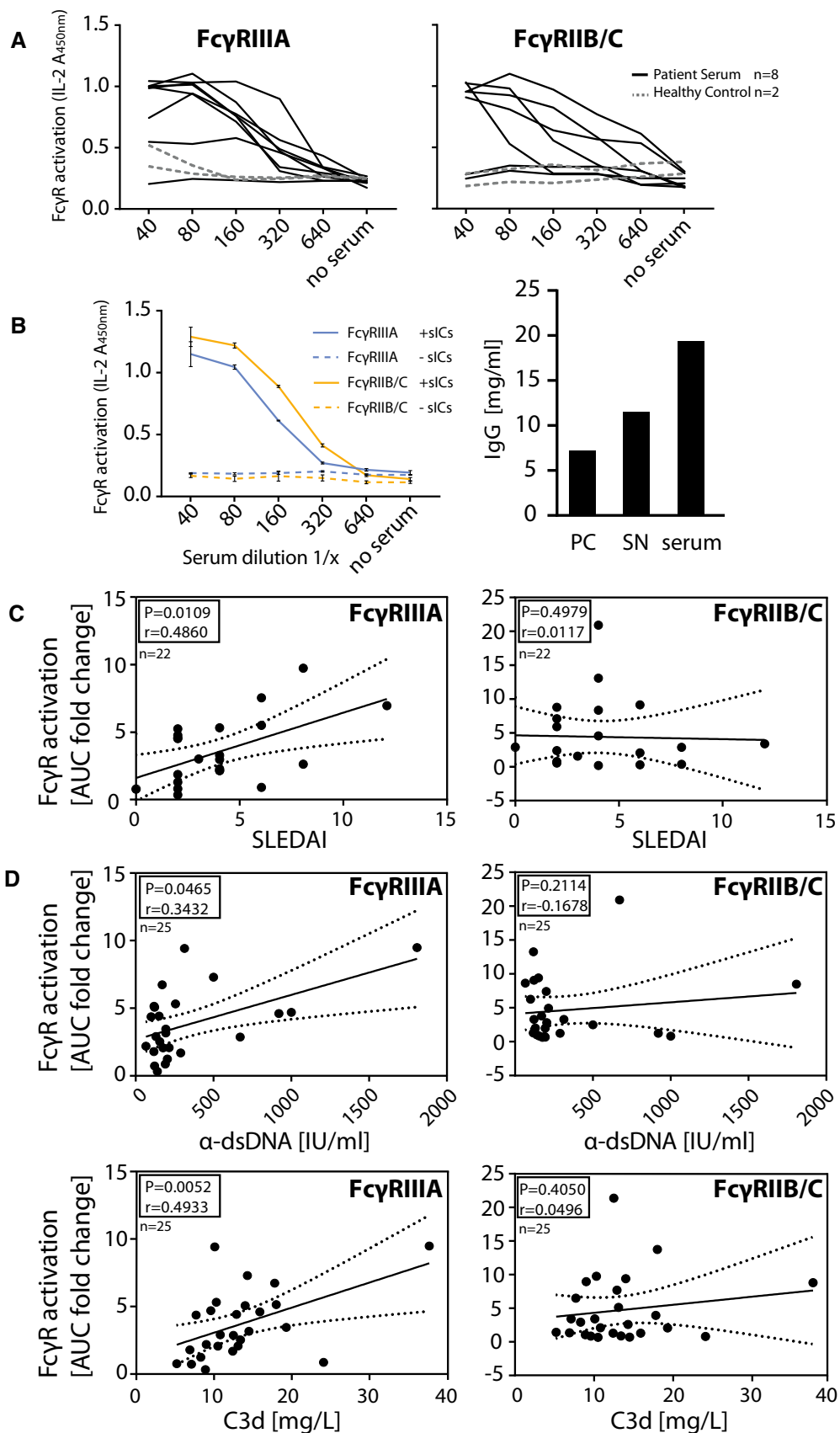


Figure 5.

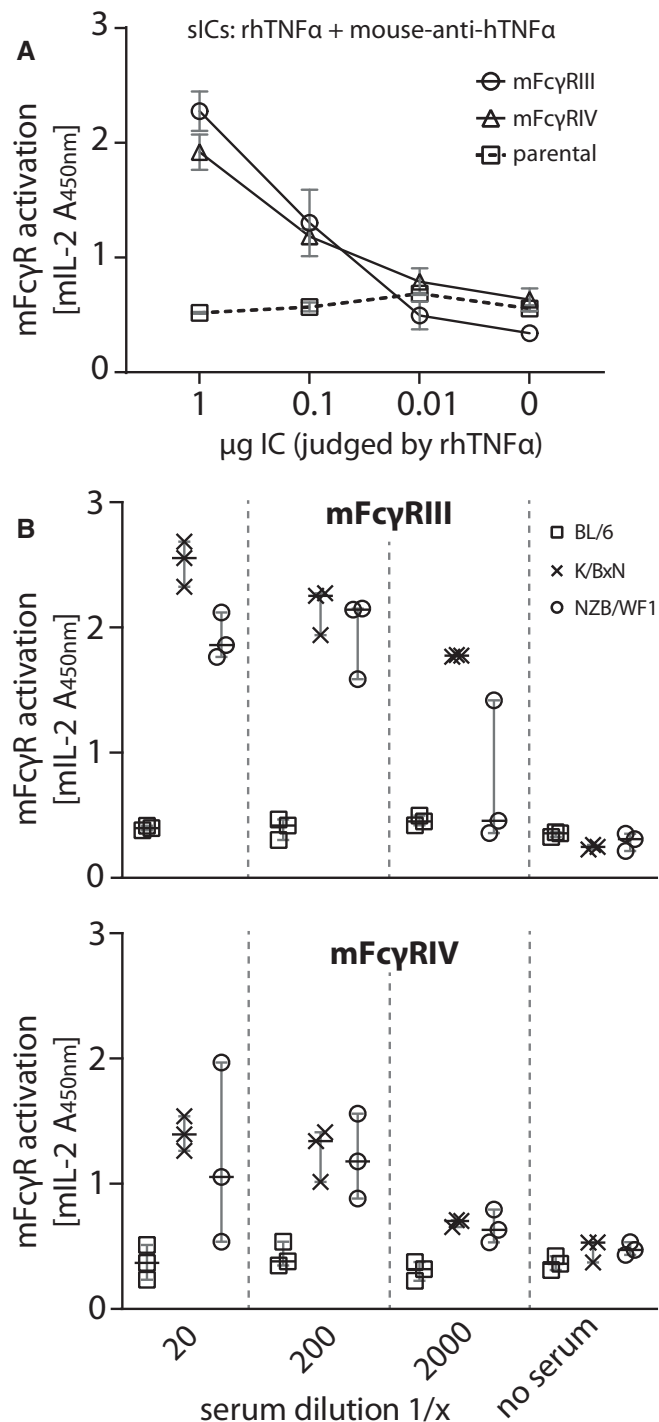


Figure 6. The reporter assay can be applied to mouse models of autoimmune disease.

A Reporter cells expressing mFc γ RIII, mFc γ RIV or parental BW5147 cells were incubated with titrated amounts of synthetic sICs generated from rhTNF- α and mouse-anti-hTNF- α at a 1:1 ratio by mass. One experiment performed in technical duplicates. Error bars indicate reproducibility as range.

B Titrations of three mouse sera per group (C57BL/6, K/BxN, or NZB/WF1) were incubated with mFc γ R reporter cells and Fc γ R activation was assessed as described above. Sera from BL/6 mice served as negative control. Each symbol represents mean values of one mouse measured in technical duplicates. Error bars = mean with SD.

conventional SLE disease markers. This is of great value as a recent analysis shows that circulating sICs and IL-6 can predict SLE activity with the higher accuracy compared to conventional clinical SLE biomarkers (Thanadetsunton *et al*, 2018). However, circulating ICs in this study were determined using a commercial C1q-binding ELISA, lacking information on immune cell bioactivity of the measured sICs. Our assay should therefore be explored as an addition to the clinician's toolbox, which may allow better disease management. Due to the scalability and high-throughput readouts, the assay can also be of use for larger prospective clinical studies in patients with autoimmune diseases such as SLE or RA, where circulating sICs have long been shown to crucially contribute to tissue damage and disease manifestations (Koffler *et al*, 1971; Zubler *et al*, 1976; Levinsky *et al*, 1977; Levinsky, 1978; Nydegger & Davis, 1980). Disease-associated, endogenous sICs can also be formed from multimeric viral and bacterial structural proteins generated during infection (Oh *et al*, 1992; Briant *et al*, 1996; Vuitton *et al*, 2020), where circulating sICs strongly impact pathogenesis (Madalinski *et al*, 1991; Wang & Ravetch, 2015).

Dynamic sIC size measurement and monitoring of bioactivity in sIC-associated diseases

The new sIC approach allowed for a simultaneous functional and biophysical assessment of the paradigmatic Heidelberg-Kendall precipitation curve (Heidelberg & Kendall, 1929, 1935). While previous work already revealed that large and small sICs differentially impact IL-6 production in PBMCs (Lux *et al*, 2013), the dynamics of Fc γ R activation resulting from constant changes in sIC size have not been explored systematically and lacked resolution of defined Fc γ R types. We analyzed synthetic sICs formed by highly pure recombinant components via AF4. Our data document that sIC size is indeed governed by antibody:antigen ratios covering a wide range of sizes up to several megadaltons. In the presence of increasing amounts of antibody or antigen deviating from an optimal antibody:antigen ratio, sIC size steadily decreases. Further, by the measurement of Fc γ R activation, we now translate physical sIC size directly to a simple but precise biological read-out. In doing so, we show that sIC size essentially tunes Fc γ R activation on and off. Thus, our new test system can not only contribute to the functional detection and quantification of clinically relevant sICs but also provides a starting point on how to avoid pathological consequences by influencing the sIC size, for example by administering and monitoring of therapeutic antibodies or recombinant antigens in controlled amounts, thus becoming relevant in clinical pharmacokinetics.

Limitations of the reporter system and conclusions

There is a wide range of factors, regulating and influencing the sIC-Fc γ R interaction. These include Fc γ -Fc γ R binding affinity and avidity (Koenderman, 2019), IgG subclass, IgG glycan profiles and genetic polymorphism (Bruhns *et al*, 2009; Pincetic *et al*, 2014; Vidarsson *et al*, 2014; Plomp *et al*, 2017), stoichiometry of antigen-antibody-ratio (Berger *et al*, 1996; Pierson *et al*, 2007; Lux *et al*, 2013), Fc γ R clustering patterns (Patel *et al*, 2019), downstream signaling (Getahun & Cambier, 2015; Bournazos *et al*, 2017), and the interaction of Fc γ R with other receptors (Vanderbruggen *et al*, 1994; Ortiz-Stern & Rosales, 2003; Douek *et al*, 2009; Urbaczek *et al*, 2014;

van Egmond *et al.*, 2015). Our assay is sensitive to amount, size and glycosylation of sICs and can readily be adapted to include more Fc γ R genotypes and polymorphisms by generation of additional reporter cell lines.

The major advancements of this reporter system include (i) a high accuracy and resolution regarding Fc γ R type-specific activation compared to traditional indirect assessment via affinity measurements, (ii) a scalable and quantifiable assay providing flexible high-throughput readouts in the nanomolar range, (iii) a reporter system sensitive to sIC size, (iv) a comprehensive panel including all human Fc γ Rs, and (v) its putative suitability as a new clinical biomarker in SLE patients and, prospectively, further patients with autoimmune diseases. In practice, the platform is suitable to be implemented into small- or large-scale screening setups in research as well as routine laboratories. Prospectively, the reporter cell approach allows for future adaptation as the cells can be equipped with alternative reporter modules to optimize the methodology for specific applications.

Materials and Methods

Cell culture

All cells were cultured in a 5% CO₂ atmosphere at 37°C. BW5147 mouse thymoma cells (BW, kindly provided by Ofer Mandelboim, Hadassah Hospital, Jerusalem, Israel) were maintained at 3×10^5 – 9×10^5 cells/ml in Roswell Park Memorial Institute medium (RPMI GlutaMAX, Gibco) supplemented with 10% (vol/vol) fetal calf serum (FCS, Biochrom), sodium pyruvate (1 \times , Gibco) and β -mercaptoethanol (0.1 mM, Gibco); 293T-CD20 (kindly provided by Irvin Chen, UCLA; Morizono *et al.*, 2010) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (vol/vol) FCS.

BW5147 cell flow cytometry

BW5147 cells were harvested by centrifugation at 900 g and RT from the suspension culture; 1×10^6 cells were stained with PE-conjugated anti-human Fc γ R mAbs (BD) or a PE-TexasRed-conjugated human IgG-Fc fragment (Rockland) for 1 h at 4°C in PBS/3%FCS. After three washing steps in PBS/3%FCS, the cells were transferred to Flow cytometry tubes (BD) and analyzed using BD LSR Fortessa and FlowJo (V10) software. Cells sorting was performed at the Lighthouse core facility of the University Hospital Freiburg using receptor staining (BD Pharmingen, PE-conjugated).

Lentiviral transduction

Lentiviral transduction of BW5147 cells was performed as described previously (Halenius *et al.*, 2011; HVan den Hoecke *et al.*, 2017; Kolb *et al.*, 2019). In brief, chimeric Fc γ R-CD3 ζ constructs (Corrales-Aguilar *et al.*, 2013) were cloned into a pUC2CL6IPwo plasmid backbone. For every construct, one 10-cm dish of packaging cell line at roughly 70% density was transfected with the target construct and two supplementing vectors providing the VSV gag/pol and VSV-G-env proteins (6 μ g of DNA each) using polyethylenimine (22.5 μ g/ml, Sigma) and Polybrene (4 μ g/ml; Merck Millipore) in a total

volume of 7 ml (2 ml of a 15-min-preincubated transfection mix in serum-free DMEM added to 5 ml of fresh full DMEM). After a medium change, virus supernatant harvested from the packaging cell line 2 days after transfection was then incubated with target BW cells overnight (3.5 ml of supernatant on 10^6 target cells), followed by expansion and pool selection using complete medium supplemented with 2 μ g/ml of puromycin (Sigma) over a one week culture period.

Human IgG suspension ELISA

1 μ g of IgG1 (rituximab in PBS, 50 μ l/well) per well was incubated on a 96 well microtiter plate (NUNC Maxisorp) pre-treated (2 h at RT) with PBS supplemented with varying percentages (v/v) of FCS (PAN Biotech). IgG1 bound to the plates was detected using an HRP-conjugated mouse-anti-human IgG mAb (Jackson ImmunoResearch).

Recombinant antigens and monoclonal antibodies to form sICs

Recombinant human (rh) cytokines TNF- α , IL-5, and VEGFA were obtained from Stem Cell technologies. Recombinant CD20 was obtained as a peptide (aa141–188, Acc# P11836) containing the binding region of rituximab (Creative Biolabs). Fc γ R-specific mAbs were obtained from Stem Cell technologies (CD16: clone 3G8; CD32: IV.3). Reverse sICs were generated from these receptor-specific antibodies using goat-anti-mouse IgG F(ab')₂ fragments (Invitrogen) in a 1:1 ratio. Pharmaceutically produced humanized monoclonal IgG1 antibodies infliximab (Ifx), bevacizumab (Bvz), mepolizumab (Mpz), and rituximab (Rtx) or IVIg (octagam 10%) were obtained from the University Hospital Pharmacy Freiburg. F(ab')₂ fragments of Ifx were generated using a Pierce F(ab')₂ preparation Kit. Mouse anti-hTNF- α (IgG2b, R&D Systems, 983003) was used to generate sICs reactive with mouse Fc γ Rs. sICs were generated by incubation of antigens and antibodies in reporter cell medium or PBS for 2 h at 37°C.

Fc γ R receptor activation assay

Fc γ R activation was measured adapting a previously described cell-based assay (Corrales-Aguilar *et al.*, 2013, 2014). The assay was modified to measure Fc γ R activation in solution. Briefly, 2×10^5 mouse BW-Fc γ R (BW5147) reporter cells were incubated with synthetic sICs or diluted serum in a total volume of 100 μ l for 16 h at 37°C and 5% CO₂. Incubation was performed in a 96-well ELISA plate (Nunc maxisorp) pre-treated with PBS/10% FCS (v/v) for 1 h at 4°C. Immobilized IgG was incubated in PBS on the plates prior to PBS/10% FCS treatment. After 4 h incubation, surface mouse CD69 expression was measured using a high throughput sampler (HTS)-FACS. Reporter cell mouse IL-2 secretion was quantified after 16 h of incubation via anti-IL-2 ELISA as described earlier (Corrales-Aguilar *et al.*, 2013).

High throughput sampler flow cytometry (HTS-FACS)

After 4 h of stimulation, 1×10^5 BW5147 reporter cells were stained with APC-conjugated anti-mCD69 (Biolegend; CD69: H1.2F3; 1:100) for 30 min at 4°C in PBS/3%FCS. Cells were transferred to a U Form 96well Microplate (Greiner 650101) and analyzed by flow cytometry

(BD Fortessa). High Throughput mode was designed within BD FACSDiva software using HTS mode with the following parameters: sample flow rate 2 $\mu\text{l/s}$, sample volume 10 μl , mixing volume 50 μl , mixing speed 200 $\mu\text{l/s}$, number of mixes two cycles and wash volume 200 μl .

BW5147 toxicity test

Cell counting was performed using a Countess II (Life Technologies) according to supplier instructions. Cell toxicity was measured as a ratio between live and dead cells judged by trypan blue staining over a 16 h time frame in a 96 well format (100 μl volume per well). BW5147 cells were mixed 1:1 with trypan blue (Invitrogen) and analyzed using a Countess II. rhTNF- α was diluted in complete medium.

NK cell activation flow cytometry

PBMC were purified from donor blood using Lymphocyte separation Media (Anprotec). Blood draw and PBMC purification from donors was approved by vote 474/18 (ethical review committee, University of Freiburg). Primary NK cells were separated from donor PBMCs via magnetic bead negative selection (Stem Cell technologies) and NK cell purity was confirmed via staining of CD3 (Biolegend, clone HIT3a), CD16 (Biolegend, clone 3G8) and CD56 (Miltenyi Biotec, clone AF12-7H3). Ninety-six well ELISA plates (Nunc Maxisorp) were pre-treated with PBS/10% FCS (v/v) for 1 h at 4°C. NK cells were stimulated in pre-treated plates and incubated at 37°C and 5% CO₂ for 4 h. Golgi Plug and Golgi Stop solutions (BD) were added as suggested by supplier. CD107a (APC, BD, H4A3) specific conjugated mAb was added at the beginning of the incubation period. Following the stimulation period, MIP-1 β (PE, BD Pharmingen), IFN γ (BV-510, Biolegends, 4SB3), and TNF- α (PE/Cy7, Biolegends, MAB11) production was measured via intracellular staining Cytokines (BD, CytoFix/CytoPerm, Kit as suggested by the supplier). 50 ng/ml PMA (InvivoGen) + 0.5 μM Ionomycin (InvivoGen) were used as a positive stimulation control for NK cell activation. After three washing steps in PBS/3%FCS, the cells were transferred to Flow cytometry tubes (BD) and analyzed using a BD FACS Fortessa and FlowJo (V10) software.

Neutrophil adhesion and activation flow cytometry

Human primary neutrophil granulocytes were isolated from whole blood of healthy donors via magnetic bead negative selection (Stemcell #19666). Ninety-six well ELISA plates (Nunc Maxisorp) were pre-treated with PBS/10% FCS (v/v) for 1 h at 4°C. Per reaction, 2×10^5 cells/ml neutrophils were stimulated with ICs in Roswell Park Memorial Institute medium (RPMI GlutaMAX, Gibco) supplemented with 10% (vol/vol) fetal calf serum (FCS, Biochrom) and incubated at 37°C and 5% CO₂ for 30 min. Adhesion and activation markers of neutrophils were measured by surface staining of CD11B (APC, Biolegend, ICRF44), CD66B (FITC, Stemcell, G10F5) and L-selectin (PE, Biolegend, DREG-56; Veen *et al*, 1998; Ilton *et al*, 1999; Lard *et al*, 1999; Khawaja *et al*, 2019). Cells were then analyzed by flow cytometry. Fc γ RII or Fc γ RIII cross-linking controls were performed by immobilization of receptor specific mAbs (Stem cell technologies, IV.3 and 3G8) before the ELISA plate was blocked.

Asymmetric flow field flow fractionation (AF4)

The AF4 system consisted of a flow controller (Eclipse AF4, Wyatt), a MALS detector (DAWN Heleos II, Wyatt), a UV detector (1,260 Infinity G1314F, Agilent) and the separation channel (SC channel, PES membrane, cut-off 10 kDa, 490 μm spacer, wide type, Wyatt). Elution buffer: 1.15 g/l Na₂HPO₄ (Merck), 0.20 g/l NaH₂PO₄ \times H₂O (Merck), 8.00 g/l NaCl (Sigma) and 0.20 g/l NaN₃ (Sigma), adjusted to pH 7.4, filtered through 0.1 μm . AF4 sequence (Vx = cross flow in ml/min): (a) elution (2 min, Vx: 1.0); (b) focus (1 min, Vx: 1.0), focus + inject (1 min, Vx: 1.0, inject flow: 0.2 ml/min), repeated three times; (c) elution (30 min, linear Vx gradient: 1.0–0.0); (d) elution (15 min, Vx: 0.0); (e) elution + inject (5 min, Vx: 0.0). A total protein mass of $17 \pm 0.3 \mu\text{g}$ (Ifx, rhTNF- α or ICs, respectively) was injected. The eluted sample concentration was calculated from the UV signal at 280 nm using extinction coefficients of 1.240 ml/(mg cm) or 1.450 ml/(mg cm) in the case of TNF- α or Ifx, respectively. For the ICs, extinction coefficients were not available and difficult to calculate, as the exact stoichiometry is not known. An extinction coefficient of 1.450 ml/(mg cm) was used for calculating the molar masses of all ICs. Especially in the case of ICs rich in TNF- α , the true coefficients should be lower, and the molar masses of these complexes are overestimated by not more than 14%. The determined molar masses for TNF- α -rich complexes are therefore biased but the observed variations in molar mass for the different ICs remain valid. The mass-weighted mean of the distribution of molar masses for each sample was calculated using the ASTRA 7 software package (Wyatt).

SLE patient cohort

Sera from patients with SLE were obtained from the Immunologic, Rheumatologic Biobank (IR-B) of the Department of Rheumatology and Clinical Immunology. Patients with SLE ($n = 25$) and healthy controls ($n = 4$) were examined. All patients met the revised ACR classification criteria for SLE. Disease activity was assessed using the SLEDAI-2K score (Gladman *et al*, 2002). C3d levels were analyzed in EDTA plasma using rocket double decker immune-electrophoresis with antisera against C3d (Polyclonal Rabbit Anti-Human C3d Complement, Agilent) and C3c (Polyclonal Rabbit Anti-Human C3c Complement Agilent) as previously described (Rother *et al*, 1993). Anti-human dsDNA antibodies titers were determined in serum using an anti-dsDNA IgG ELISA kit (diagnostik-a GmbH).

Patient serum IC precipitation

For PEG precipitation human sera were mixed with PEG 6000 (Sigma-Aldrich) in PBS at a final concentration of 10% PEG 6000. After overnight incubation at 4°C, ICs were precipitated by centrifugation at 2,000 g for 30 min at 4°C, pellets were washed once with PEG 6000 and then centrifuged at 2,000 g for 20 min at 4°C. Supernatants were harvested and precipitates resuspended in pre-warmed PBS for 1 h at 37°C. IgG concentrations of serum, precipitates, and supernatants obtained after precipitation were quantified by Nanodrop (Thermo Scientific™) measurement.

The paper explained

Problem

Soluble immune complexes (sICs) in the blood or certain body fluids are thought to be major drivers of immunopathology in certain autoimmune diseases or infections. sICs are recognized by Fc-gamma receptors (FcγRs) on several immune cells, leading to their activation and subsequent tissue inflammation. While assays measuring individual features such as binding affinity or concentration of sICs are available, an assay detecting actual bioactivity of circulating ICs in clinical or experimental settings was missing.

Results

We developed an assay that utilizes FcγR expressing reporter cells to detect sIC mediated bioactivity. The reporter panel includes all human FcγRs and allows for the evaluation of individual receptor activation. The assay is quantitative, flexible by providing alternative readouts, and scalable from single samples to high-throughput methodology. The assay was successfully employed to detect sICs in the serum of patients with SLE and the measured sIC bioactivity was consistent with clinical and conventional diagnostic markers of disease severity.

Impact

Our methodology presents a useful addition to the diagnostic and experimental toolbox of immunologists. The assay provides a unique sensitivity to sIC size, a feature that is important to sIC bioactivity but currently not addressed by available methodology. With its scalability and unparalleled sensitivity in the nanomolar range, the assay could detect the onset of autoimmunity flares or immunopathology in infection at a very early stage.

Mice and models

Lupus-prone (NZBxNZW)F1 mice (NZB/WF1) were generated by crossing NZB/BINJ mice with NZW/LacJ mice, purchased from The Jackson Laboratory. KRNtg mice were obtained from F. Nimmerjahn (Universität Erlangen-Nürnberg) with the permission of D. Mathis and C. Benoist (Harvard Medical School, Boston, MA), C57BL/6 mice (BL/6) and NOD/ShiLtJArc (NOD/Lt) mice were obtained from the Charles River Laboratories. K/BxN (KRNtgxNOD)F1 mice (K/BxN) were obtained by crossing KRNtg mice and NOD/Lt mice. All mice were housed in a 12-h light/dark cycle, with food and water *ad libitum*. Mice were euthanized and blood collected for serum preparation from 16 weeks old BL/6 animals, from 16 weeks old arthritic K/BxN animals and from 26 to 38 weeks old NZB/WF1 mice with established glomerulonephritis.

Ethics

Biobanking and the project were approved by the local ethical committee of the University of Freiburg (votes 507/16 and 624/14). All patients who provided blood to the biobank had provided written informed consent. Ethical Statement: The study was designed in accordance with the guidelines of the Declaration of Helsinki (revised 2013) and conformed to the principles set out in the Department of Health and Human Services Belmont Report. Animal experiments were approved by the local governmental commission for animal protection of Freiburg (Regierungspräsidium Freiburg, approval no. G16/59 and G19/21).

Statistical analyses

Statistical analyses were performed using Graphpad Prism software (v6) and appropriate tests. Multiple comparison by ANOVA. No randomization was used. No blinding was done. Sample size: Primary NK cells and Neutrophils were isolated from three or four healthy donors to enable statistical analysis and ensure reproducibility across donors. 25 SLE patients were compared to four healthy control sera. Three individual mice from each genetic background were chosen as an adequate sample size to show clear trends.

Data availability

All data associated with this study are present in the paper or in the Expanded View.

Expanded View for this article is available online.

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Author contributions

Conceived and designed the experiments: PK, HC, AM-P, MHO. Performed the experiments: PK, HC, MHO, MHU, AM-P, US. Analyzed the data: PK, HC, MHO, AM-P, US. Contributed/reagent/sample material: REV, NC. Writing and original draft preparation: PK, HC. Review and editing: HH, REV, PK.

Conflict of interest

The authors declare that they have no conflict of interest.

For more information

- <https://www.uniklinik-freiburg.de/virologie-en/research/research-teams/hartmut-hengel-team.html>
- <https://www.uniklinik-freiburg.de/virologie-en/research/research-teams/philipp-kolb-team.html>

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