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Impact of Immune Complex Size and Glycosylation on IgG Binding to Human FcγRs

Anja Lux,* Xiaojie Yu,[†] Chris N. Scanlan,[†] and Falk Nimmerjahn*

IgG molecules are widely used as therapeutic agents either in the form of intact Abs or as Fc fusion proteins. Although efficient binding of the IgG Fc fragment to cellular FcγRs may be essential to achieve a high cytolytic activity, it may be advantageous for other applications to limit or abolish this interaction. Genetic or biochemical approaches have been used to generate these non-FcγR-binding IgG variants. By using soluble versions of FcγRs and monomeric versions of these altered IgG molecules, it was demonstrated that these IgG variants no longer bind to FcγRs. Importantly, however, these assays do not reflect the physiologic interaction of IgG with low-affinity cellular FcγRs occurring in the form of multimeric immune complexes. In this study, we investigated how the size of an immune complex can affect the interaction of normal and various versions of potentially non-FcγR-binding IgG variants with cellular FcγRs. We show that neither the D265A mutation nor EndoS treatment resulting in IgG molecules with only one *N*-acetylglucosamine and a fucose residue was fully able to abolish the interaction of all IgG subclasses with cellular FcγRs, suggesting that IgG subclass-specific strategies are essential to fully interfere with human FcγR binding. *The Journal of Immunology*, 2013, 190: 4315–4323.

Antibodies of the IgG isotype are critical for a fully functional immune system. Cytotoxic Abs aiming at depletion or killing of malignant or autoreactive cell populations have been used successfully in cancer therapy and more recently in the treatment of autoimmune diseases (1–3). Studies over the last years emphasized that the efficient interaction of cytotoxic Abs with cellular FcγRs expressed widely on innate immune effector cells, including NK cells, neutrophils, monocytes, and macrophages, is critical for IgG activity in vivo (4–6). Thus, mice deficient in all or select FcγRs showed an impaired IgG activity and were unable to deplete target cell populations. Moreover, deletion of the inhibitory FcγRIIB, which is coexpressed with activating FcγRs on the majority of innate effector cells, enhanced cytotoxic IgG activity, providing the basis for the concept that therapeutic IgG may be optimized by generating Abs with improved binding to FcγRs (7–9). Evidence that this concept may be relevant for human IgG activity was afforded by studies showing that patients carrying activating FcγR alleles with higher affinity for therapeutic Abs responded better to therapy (10–13). These studies also revealed that the low-affinity human FcγRs, FcγRIIA and FcγRIIIA, may be particularly

relevant for human IgG activity in vivo. In addition to cytotoxic IgG, either intact Abs or Fc fusion proteins are being used increasingly to neutralize proinflammatory cytokines in patients with autoimmune diseases, such as rheumatoid arthritis (14). Moreover, Ab–Ag fusion proteins can be used to target the Ag of choice to phagocytic receptors on dendritic cells either for the induction of immunity or tolerance (15, 16). For these applications, it may be critical to use IgG Fc fragments with abrogated binding to cellular FcγRs. Although the generation of F(ab')₂ fragments is one possible solution to this problem, it results in a greatly reduced half-life of the Ab because it does not bind to the neonatal FcRn (17, 18). Thus, strategies have been developed to abrogate binding to the family of canonical FcγRs while maintaining the interaction with the neonatal FcRn. The most frequently used approaches include deglycosylation of the IgG Fc fragment by treatment with endoglycosidases, such as EndoS (which removes most of the *N*-glycan, leaving the protein-proximal *N*-acetylglucosamine, along with any branched core-fucose residue, attached to Asn²⁹⁷ of the Fc) or full deglycosylation, using protein *N*-glycanases, such as peptide:*N*-glycosidase F (PNGase F) (which removes the entire *N*-glycan). Alternatively, mutations can be introduced into the IgG backbone abrogating IgG glycosylation (e.g., by exchanging the asparagine 297 residue with an alanine residue [N297A]). In a similar manner, replacing the aspartate residue at position 265 with an alanine residue (D265A) in human IgG1 and several mouse IgG subclasses also abrogates FcγR binding (9, 19, 20). To demonstrate the absence of FcγR-binding activity, protein–protein interaction analyses, such as surface plasmon resonance, using soluble FcγRs and monomeric IgG are widely used. Although this type of analysis is of great use to quantitatively assess changes in affinity, it does not reflect the interaction of IgG with cellular FcγRs in vivo. With the exception of the high-affinity FcγRI, all other low-affinity FcγRs achieve functional interactions with IgG in the form of multimeric IgG immune complexes (ICs) (4, 6, 21). Depending on the abundance or availability of the Ag or Ab, the size of ICs can vary and, therefore, may affect binding to cellular FcγRs. Therefore, it is essential to ensure that IgG variants with a decreased affinity for soluble FcγRs also show an abrogated binding to cellular FcγRs if they appear in the form of an IC. To

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Abbreviations used in this article: CHO, Chinese hamster ovarian; EndoS, endoglycosidase S; IC, immune complex; PEG, polyethylene glycol; PNGase F, peptide:*N*-glycosidase F; TNP, 2-,4-,6-trinitrophenyl; TNP-4-BSA, BSA coupled to four 2-,4-,6-trinitrophenyl groups; TNP-26-BSA, BSA coupled to 26 2-,4-,6-trinitrophenyl groups.

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address this question in a systematic and quantitative manner, we generated ICs of different sizes with all human IgG subclasses and with widely used non-FcγR-binding IgG variants. Surprisingly, this analysis revealed that, in certain combinations of FcγR alleles and human IgG subclasses, the interaction of certain non-FcγR-binding IgG variants was not abrogated if present in a large IC. These results suggest that, depending on the FcγR alleles present in the patient and depending on the size of the generated IC, only certain IgG variants are completely deficient in FcγR binding.

Materials and Methods

Abs and cell lines

IC binding to human FcγRs was analyzed using Chinese hamster ovarian (CHO) cells stably expressing human FcγRs as described (22). CHO cell lines expressing human FcγRIA, FcγRIIB, FcγRIIA-131R, and FcγRIIA-131H were generated in our laboratory by Anja Lux. CHO cell lines stably expressing human FcγRIIA-158F and FcγRIIA-158V were kindly provided by M. Daeron (Institute Pasteur, Paris, France). Human FcγR expression on CHO cell lines was confirmed in each experiment by staining with FITC-coupled Abs directed against FcγRIA (CD64; clone 10.1; BD Pharmingen), FcγRIIA/IIB (CD32; clone 3D3; BD Pharmingen), and FcγRIIA (CD16; clone 3G8; BD Pharmingen). Human FcγRIIB (CD32B) expression was detected with the Ab 2B6 generously provided by Jeffrey Stavenhagen (Macrogenics) and labeled with Alexa Fluor 647 (Invitrogen).

Cloning and production of human anti-2,4,6-trinitrophenyl IgG

Human anti-2,4,6-trinitrophenyl (TNP) variable regions were cloned from cDNA libraries isolated from the 7B4 hybridoma cell line (kindly provided by Birgitta Heyman, University of Uppsala, Uppsala, Sweden), fused to human IgG constant regions by PCR, and produced by transient transfection in HEK293T cells in protein-free medium, followed by purification from the supernatant via protein G, as described (23). Point mutations at position asparagine 297 (N297A) and aspartate 265 (D265A) were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's instructions.

Analysis of TNP-BSA-binding capacity

Ag binding of human anti-TNP IgG subclasses was compared by ELISA. Briefly, 1 μg/ml BSA coupled with either 4 (TNP-4-BSA) or 26 (TNP-26-BSA) TNP molecules (Biosearch Technologies) was coated on microtiter plates in 0.05 M carbonate/bicarbonate buffer (pH 9.6) and incubated with 10 ng/ml anti-TNP IgG in PBS/1% BSA, followed by detection of bound IgG using an HRP-conjugated anti-human IgG F(ab')₂ fragment at 100 ng/ml (Bethyl Laboratories).

Glycan analysis

Oligosaccharides were released from target glycoproteins with peptide-N-glycosidase F (New England BioLabs) from Coomassie blue-stained NuPAF2455561GE gels (24). Excised bands were washed five times alternately with acetonitrile and deionized water and rehydrated with 3000 U/ml aqueous PNGase F solution. After 12 h of incubation at 37°C, the enzymatically released N-linked glycans were eluted with water. Samples were analyzed by MALDI-TOF mass spectrometry with a Shimadzu AXIMA MALDI TOF/TOF mass spectrometer (Kratos Analytical, Manchester, U.K.) equipped with delayed extraction and a nitrogen laser (337 nm). Samples were cleaned on a Nafion 117 membrane (Sigma-Aldrich) and then prepared for MALDI-mass spectrometry by adding 0.5 μl aqueous solution of the glycans to the matrix solution (0.3 μl solution of 2,5-dihydroxybenzoic acid in acetonitrile/water; 1:1, v/v) on the stainless steel target plate and allowing it to dry at room temperature. The sample/matrix mixture was then recrystallized from ethanol.

IC preparation and polyethylene glycol precipitation

ICs were generated by coinubation of 10 μg/ml human anti-TNP IgG and 5 μg/ml TNP-coupled BSA for 3 h with gentle shaking at room temperature. The relative size of TNP-4-BSA and TNP-26-BSA ICs was analyzed by polyethylene glycol (PEG) precipitation (25). Briefly, ICs were mixed with PEG 6000 in PBS at a final concentration of 0–20% PEG 6000. After overnight incubation at 4°C, ICs were precipitated by centrifugation at 2000 × g for 30 min at 4°C and washed once with PEG 6000, followed by precipitation at 2000 × g and resuspension by shaking in prewarmed PBS for 1 h at 37°C. ICs precipitating at the respective PEG concentrations were detected by ELISA using the Human IgG ELISA Quantitation Kit

(Bethyl Laboratories), according to the manufacturer's instructions. The amount of IgG present in the different fractions was normalized to the input before PEG precipitation and is shown as arbitrary units.

Analysis of IC binding to cellular human FcγRs

ICs were incubated with 100,000 CHO cells stably expressing human FcγRs, human B cells (cell line LCL1.11), or 200,000 human peripheral blood leukocytes for 1 h under gentle shaking at 4°C. Bound ICs were detected by flow cytometry on a FACSCalibur or FACSCanto II (both from BD Biosciences) using a PE-conjugated goat anti-human IgG F(ab')₂ fragment at 0.5 μg/ml (Jackson ImmunoResearch Laboratories). Human peripheral blood leukocytes were purified by RBC lysis and stained with FITC-conjugated anti-CD56 (clone NCAM16.2; BD Pharmingen) and allophycocyanin-conjugated anti-CD33 (clone P67-6; BD Pharmingen) Abs to detect IC binding to NK cells and myeloid cells, respectively. Binding to granulocytes was detected by gating SSC^{high} cells. Data were analyzed with Flow Cytometry Analysis Software (FlowJo) or FACSDiva Software. To study binding of aglycosylated or partially glycosylated IgG subclasses to FcγRs, IgG was enzymatically deglycosylated with recombinant endoglycosidase S or PNGase F (New England BioLabs) before the generation of ICs. Briefly, Abs were incubated with 50 U/μg IgG PNGase F or 1 μg endoglycosidase S (EndoS)/100 μg IgG overnight at 37°C. Complete deglycosylation was confirmed by PAGE under reducing conditions and subsequent staining with Coomassie brilliant blue or by lectin blotting, as previously described (26). IC binding to human peripheral blood leukocytes was blocked by preincubation of cells with anti-FcγRI (clone 10.1), anti-FcγRIIA/B (clone AT10; provided by M. Glennie, University of Southampton, Southampton, U.K.), and anti-FcγRIII (clone 3G8) at 10 μg/ml. Data were analyzed with Flow Cytometry Analysis Software (FlowJo).

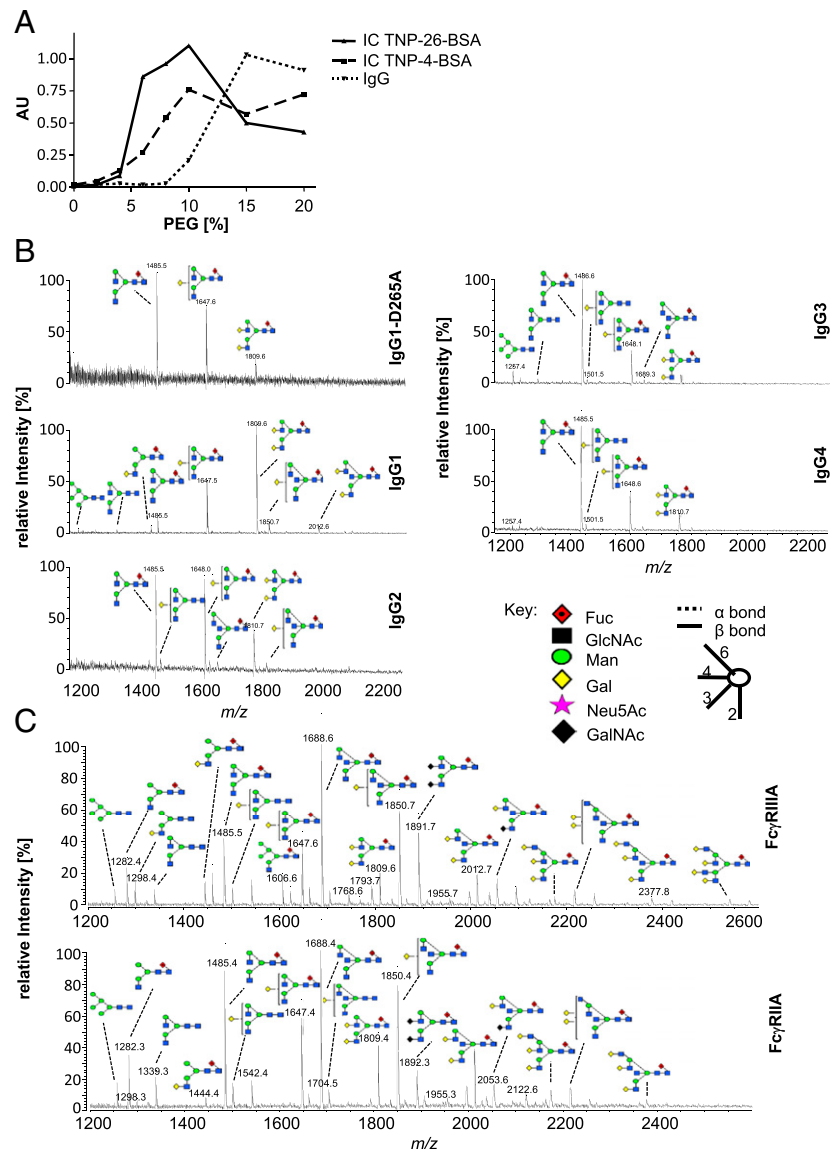
Analysis of immune cell activation after IC binding

Human peripheral blood leukocytes were purified by RBC lysis and incubated with TNP-BSA ICs overnight at 37°C and 5% CO₂ in RPMI 1640 containing 10% FCS, 1% penicillin/streptomycin, 1% glutamine, 1% nonessential amino acids, and 1% sodium pyruvate. Cell culture supernatants were analyzed by cytokine bead array for the presence of IL-6 using the BD CBA Human Soluble Protein Flex Set Assay (BD Biosciences), according to the manufacturer's instructions.

Results

Generation of human IgG subclass ICs with different sizes

To study the interaction of ICs with cellular FcγRs under defined conditions *in vitro*, we used CHO cells stably transfected with human FcγRIA, the FcγRIIA-131H and FcγRIIA-131R allele, the FcγRIIA-158V and FcγRIIA-158F allele, or FcγRIIB, as described previously (22, 27). Human IgG ICs of different sizes were generated by incubating human IgG subclass switch variants of the murine TNP-specific Ab 7B4 with BSA coupled to either 4 TNP groups (TNP-4-BSA) or 26 TNP groups (TNP-26-BSA) (28). Analysis of IC size by PEG precipitation verified that incubation of anti-TNP IgG subclasses with TNP-26-BSA resulted in the generation of a larger IC compared with TNP-4-BSA or monomeric IgG (Fig. 1A) (25). Furthermore, MALDI-TOF analysis verified that, irrespective of the IgG subclass, our rIgG preparations contained high levels of branching fucose residues and were largely in the IgG-G0 or IgG-G1 form, consistent with previous reports of mouse Abs produced in HEK293 cells (Fig. 1B) (29). In the serum, IgG-G0 and IgG-G1 glycoforms represent ~70% of the total IgG glycovariants, suggesting that our experimental system may be valid for the majority of the IgG glycovariants present *in vivo* (30). As described previously for another IgG-D265A variant produced in CHO cells, the IgG1-D265A mutant that we produced in HEK293 cells showed a shift toward more terminal galactose residues (31). Thus, production of Abs in HEK293 cells results in a glycosylation pattern (notably fucosylation levels) sufficiently close to that of native serum so as not to affect FcγR affinity. In addition, we determined the glycosylation pattern of the FcγRIIA and FcγRIIA variants. As shown in Fig. 1C, this identified a very complex array of sugar structures attached to the various N-linked



glycosylation sites in these receptors, consistent with previous reports (32–35), again indicating that these recombinant glycoproteins were correctly expressed and folded.

Effect of IC size on IgG subclass-specific binding to human cellular FcγRs

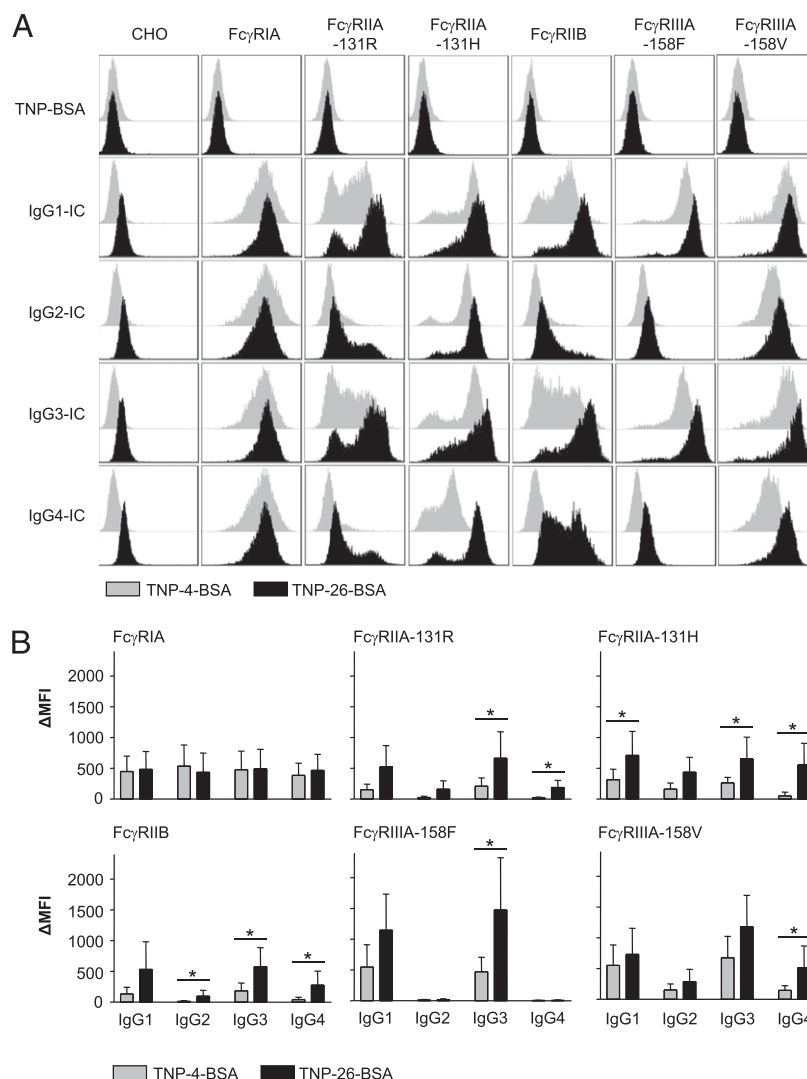
Having established the experimental system, we analyzed how the size of different human IgG subclass ICs affects binding to human FcγRs stably expressed on CHO cells, as described (22). As shown in Fig. 2, virtually no binding of ICs to untransfected CHO cells was observed, whereas varying degrees of binding were noted depending on the IgG subclass, the respective human FcγR, and the size of the IC. As expected, the least effect of IC size was observed for the high-affinity FcγRIA, which is the only receptor capable of appreciable binding to monomeric IgG1, IgG3, and IgG4 (22, 36) (Fig. 2). Interestingly, however, TNP-4-BSA and TNP-26-BSA ICs of all human subclasses, including IgG2, which does not bind to FcγRIA as a monomer or as an anti-F(ab')₂-aggregated IC (22), could bind to FcγRIA equally well. In contrast, the size of the IC did affect the level of binding to the low-affinity FcγRs. Thus, a better binding of larger IgG1, IgG3, and IgG4 ICs was observed for both alleles of FcγRIIA. In a similar fashion, IgG2-, IgG3-, and IgG4-TNP-26-BSA ICs bound better to FcγRIIB.

A smaller effect of IC size was obtained for binding to the two allelic variants of human FcγRIIA: only IgG3-TNP-26-BSA complexes showed a higher level of binding to FcγRIIA-158F, and IgG4-TNP-26-BSA complexes demonstrated increased binding to FcγRIIA-158V. Of note, the IgG4 subclass, usually considered to have low or no binding to FcγRIIA, FcγRIIB, and FcγRIIA, showed particularly enhanced binding to low-affinity FcγRs if present as a large IC (Fig. 2). Moreover, we observed an effect of certain FcγR alleles on IC binding, demonstrated by human IgG2 and IgG4 binding to the FcγRIIA-158V allele, but not to the FcγRIIA-158F allele, irrespective of IC size.

Effect of IC size on the interaction of non-Fc-binding IgG1 variants to cellular FcγRs

Having demonstrated that the size of an IC can convert human IgG subclasses considered to have low or no affinity for individual human FcγRs to ligands of these receptors, we investigated whether IC size can also overcome the abrogated binding of so-called “non-FcγR-binding IgG1 mutants.” One frequently introduced mutation to prevent mouse and human IgG from binding to FcγRs is to exchange the aspartate residue at position 265 for an alanine residue (D265A) (9, 19, 20, 23, 31). As shown in Fig. 3A, introduction of the D265A mutation in the IgG1 subclass variant of the anti-TNP

FIGURE 2. Binding of human IgG TNP-4-BSA and TNP-26-BSA ICs to human FcγRs. ICs were generated by coincubation of 10 μg/ml of the respective human anti-TNP IgG subclasses with 5 μg/ml TNP-4-BSA or TNP-26-BSA, followed by incubation with CHO cells expressing either none (CHO) or one individual human FcγR. Bound ICs were detected by flow cytometry using a PE-conjugated goat anti-human IgG F(ab')₂ fragment. **(A)** Representative graphs of human IgG subclass TNP-4-BSA and TNP-26-BSA ICs binding to the CHO cell lines expressing the indicated human FcγRs. One of four independent experiments is shown. **(B)** Quantification of human IgG subclass TNP-4-BSA and TNP-26-BSA IC binding to CHO cells expressing the indicated human FcγRs in four independent experiments. Background binding of the ICs to CHO cells expressing no human FcγR was subtracted from the mean fluorescence intensity (MFI) obtained for binding to CHO cells expressing individual human FcγRs (Δ MFI $\pm$ SD). * p < 0.05, Kruskal–Wallis test.



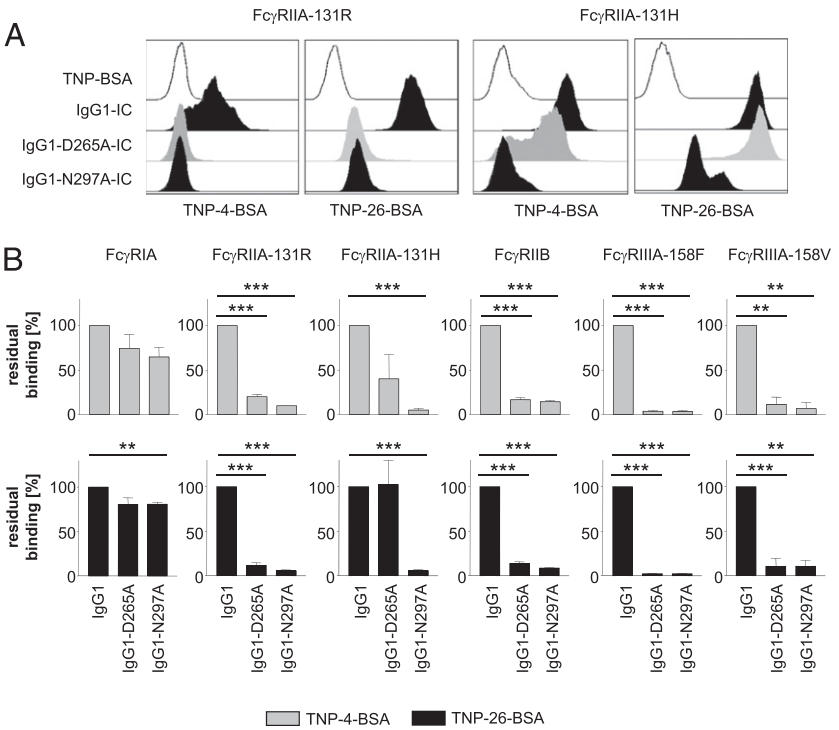
Ab dramatically reduced the interaction of the small and large ICs with the FcγRIIA-131R allele. Similarly, the binding to FcγRIIB and both of the FcγRIIA alleles was impaired (Fig. 3B). However, binding to the FcγRIIA-131H allele was still clearly detectable for the small IC and was unchanged compared with normal IgG1 when present in the large IC. The least effect of the D265A mutation was observed for IgG1 binding to the high-affinity receptor; both the small and the large IgG1-D265A IC were indistinguishable from normal IgG1 (Fig. 3B). In addition to the IgG1-D265A variant, the exchange of the asparagine residue at position 297 to an alanine (N297A) resulted in impaired binding to mouse and human FcγRs by deleting the acceptor site for attachment of the sugar moiety linked to both CH2 domains of the IgG molecule (37, 38). Compared with the IgG1-D265A mutation, the IgG1-N297A variant was incapable of binding to the low-affinity human FcγRs, regardless of IC size (Fig. 3, Table I). However, as for the D265A variant, only a minor effect of the N297A mutation on binding to the high-affinity FcγRIA was detectable, consistent with previous reports demonstrating that aglycosylated human IgG3 retained its capacity to trigger the phagocyte respiratory burst (39).

Effect of PNGase F-mediated IgG deglycosylation on the interaction with human FcγRs

To further study the role of the IgG sugar moiety for binding to human FcγRs, we treated all anti-TNP IgG subclass variants with

PNGase F, resulting in complete removal of the sugar side chain (confirmed by the loss of lens culinaris agglutinin binding; Supplemental Fig. 1). Consistent with the data for the IgG1-N297A variant, the PNGase F-treated IgG1 showed a strong reduction of binding to all low-affinity FcγRs but maintained binding to the high-affinity FcγRIA, independent of IC size (Fig. 4A). Comparable results were obtained for all other subclasses with respect to binding to the low-affinity FcγRs (Fig. 4B–D, Table II). In contrast, IgG subclass-specific effects could be observed for PNGase F-treated IgG with respect to binding to the high-affinity FcγRIA. Although deglycosylation of IgG1 or IgG3 resulted in a very modest reduction in binding, more drastic effects were seen for IgG2 and IgG4. A 50% reduction in binding was observed, which, in the case of IgG4, was influenced slightly by the size of the IC. These results are consistent with the affinities of these subclasses to the high-affinity receptor: IgG1 and IgG3 had the highest affinity, followed closely by IgG4 (22). With respect to IgG2, our data suggest that it has the least affinity for FcγRIA; however, it is sufficient to interact with FcγRIA in the form of an IC. A notable exception between the genetically (N297A) and the biochemically (PNGase F) generated aglycosyl variants is that the PNGase F-treated large IgG1 (and also the IgG3) IC only showed a 50% reduction in binding to the FcγRIIA-158V allele. A possible, but speculative, explanation for this result is that PNGase F converts the asparagine residue to aspartate, which may affect the local secondary structure in a region close to the FcγR binding site by its charge.

FIGURE 3. Influence of IC size on binding of mutated IgG1 to human FcγRs. **(A)** Graphs of TNP-4-BSA and TNP-26-BSA binding to CHO cell lines expressing the FcγRIIA-131R or the FcγRIIA-131H allele either without Abs (TNP-BSA) or as ICs with TNP-specific IgG1, the IgG1-D265A variant, or the IgG1-N297A variant. One of three representative experiments is shown. **(B)** Quantification of TNP-specific IgG1, IgG1-D265A, and IgG1-N297A binding to CHO cells expressing the indicated human FcγRs as an IC with TNP-4-BSA or TNP-26-BSA. Residual binding of TNP-specific IgG1-D265A and IgG1-N297A ICs to FcγR expressing CHO cells normalized to the binding of nonmutated TNP-specific IgG1 ICs (set to 100%) ± SD from three independent experiments. ***p* < 0.01, ****p* < 0.001, Kruskal–Wallis test and subsequent post hoc tests.



Effect of EndoS-mediated IgG deglycosylation on the interaction with human FcγRs

In addition to the complete removal of the sugar moiety with PNGase F, another endoglycosidase derived from *Streptococcus pyogenes* (EndoS) was demonstrated to reduce the binding of human, rabbit, and mouse IgG to FcγRs (26, 40–42). In contrast to PNGase F, EndoS treatment retains the penultimate *N*-acetylglucosamine residue and the branching fucose residue attached to it (Supplemental Fig. 1). Previous studies in mice and with human IgG subclasses suggest that, despite the efficient removal of the sugar moiety of all IgG subclasses, the affinity of mouse IgG2a and human IgG2 for FcγRs does not seem to be impaired (26, 41). Indeed, EndoS-treated mouse IgG2a remained fully active in a model of immunothrombocytopenia, whereas the platelet-depleting activity of IgG1 and IgG2b subclass switch variants was completely abrogated (26). To study how IC size influences the capacity of EndoS treatment to impair the IgG–FcγR interaction, we treated all human anti-TNP IgG subclasses with EndoS, resulting in an efficient removal of the sugar moiety, as indicated by a reduction in molecular weight and loss of lens culinaris agglutinin binding, as demonstrated previously (Supplemental Fig. 1B) (41). Compared with PNGase F treatment, EndoS-digested IgG2 and IgG4 subclasses showed either no or only a very modest reduction in binding to the high-affinity FcγRIA for the small and large IC (Fig. 4). All IgG subclasses showed a diminished binding to the low-affinity receptors FcγRIIA-131R, FcγRIIB, and FcγRIIA-158F, even in the form of a large IC. In contrast, IC size dramatically influenced

EndoS-treated IgG binding to FcγRIIA-131H (Fig. 4). Although EndoS treatment abolished or strongly reduced binding of the small IC of all IgG subclasses, IgG1, IgG2, and IgG3 bound at wild-type levels to this FcγRIIA allele if present as a large IC (Table II). With respect to the FcγRIIA-158V allele, IgG subclass-specific, as well as IC size-specific, effects were observed. Only a partial impairment of IgG1 and IgG3 binding, especially for the binding of the small IC, was observed, whereas the large IC bound at wild-type levels. However, IgG2 and IgG4 binding to FcγRIIA-158V was abolished, independent of IC size. In contrast to previous data suggesting that the interaction of monomeric IgG2 with soluble human FcγRs is not impaired by EndoS treatment, our results clearly indicate that EndoS-treated IgG2 shows a reduced or diminished interaction with low-affinity FcγRs. Overall, these data indicate that the presence of a core *N*-acetylglucosamine residue (retained after EndoS but not PNGase F digestion) provides a residual affinity for FcγR. This is consistent with known structures of IgG1 Fc in which the C'E loop, critical for receptor binding, is displaced in the structure of a nonglycosylated Fc (PDB 3S7G), whereas the structure of an endoglycosidase-treated Fc reveals a loop structure more closely resembling wild-type Fc (43, 44). These observations provide a structural basis for the differential effects of PNGase F and EndoS on IgG–Fc–FcγR interactions.

IgG1 IC binding to human cells

One area of concern when using a recombinant model system, such as CHO cells stably expressing individual human FcγRs, is that the

Table I. Influence of IC size on human IgG1-D265A and IgG1-N297A binding to human FcγRs

Ab Variant	FcγRIA		FcγRIIA-131R		FcγRIIA-131H		FcγRIIB		FcγRIIA-158F		FcγRIIA-158V	
	TNP-4	TNP-26	TNP-4	TNP-26	TNP-4	TNP-26	TNP-4	TNP-26	TNP-4	TNP-26	TNP-4	TNP-26
IgG1	++	++	+	++	++	+++	+	++	++	+++	++	+++
D265A	++	++	–	–	+	+++	–	–	–	–	–	–
N297A	++	++	–	–	–	–	–	–	–	–	–	–

The binding of TNP-specific IgG1, IgG1-D265A, and IgG1-N297A ICs to human FcγRs was graded as no binding (–), weak binding (+), good binding (++), and strong binding (+++).

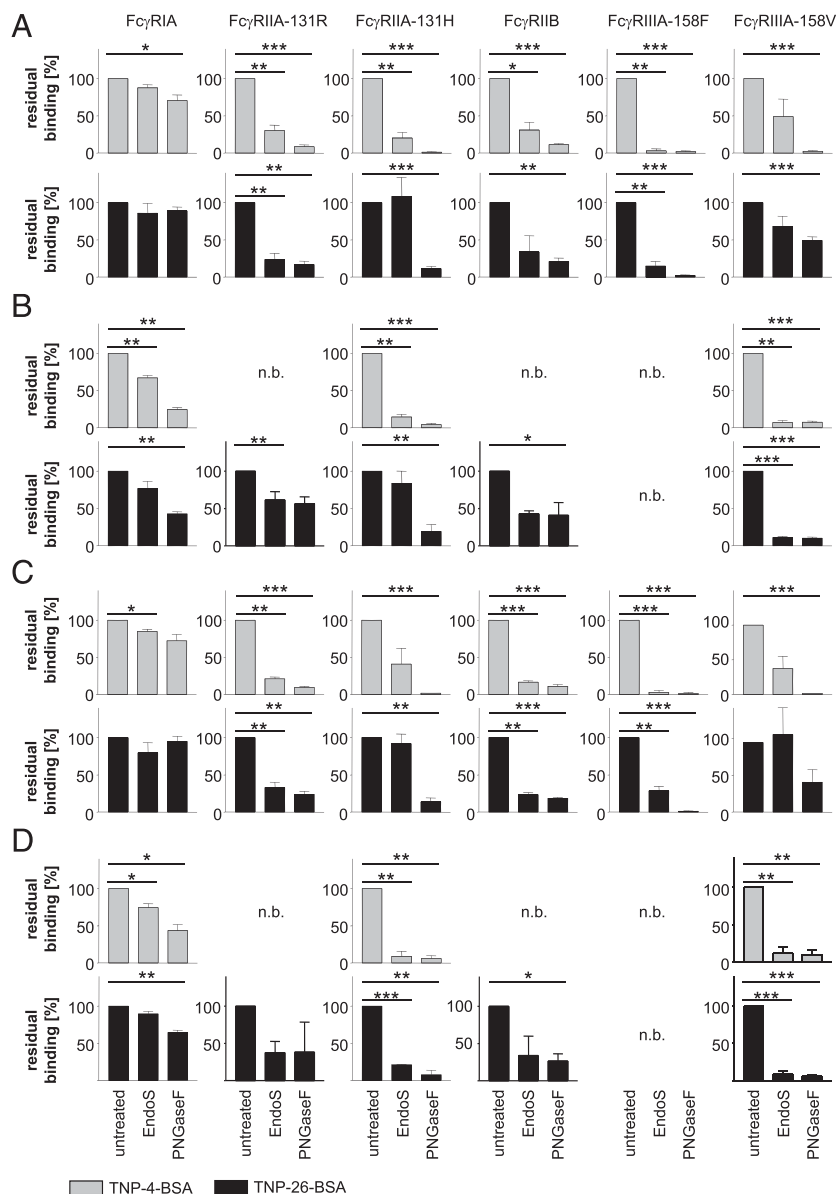


FIGURE 4. Influence of IC size on binding of PNGase F- or EndoS-treated human IgG subclasses to human FcγRs. Residual binding capacity of TNP-4-BSA and TNP-26-BSA in complex with human IgG1 (**A**), IgG2 (**B**), IgG3 (**C**), or IgG4 (**D**) either left untreated or treated with PNGase F or EndoS to deglycosylate the respective IgG subclass. Bar graphs indicate the residual IC binding normalized to untreated human IgG IC binding (set to 100%) of three independent experiments $\pm$ SD. Please note that certain IgG subclass–FcγR combinations showed no binding (n.b.) and, thus, could not be evaluated by this experimental approach. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Kruskal–Wallis test and subsequent post hoc tests.

data may not be representative for human cells expressing physiological levels of FcγRs. Therefore, we repeated some of the experiments with a human B cell line and with primary human

PBMCs. Because B cells solely express FcγRIIB, this is an ideal system to exclusively study IC binding to this receptor. As shown in Fig. 5A, we could fully recapitulate the data obtained in the

Table II. Influence of IC size on human IgG subclass binding to human FcγRs after deglycosylation with PNGase F or EndoS

IgG Subclass	Glycosidase Treatment	FcγRIA		FcγRIIA-131R		FcγRIIA-131H		FcγRIIB		FcγRIIIA-158F		FcγRIIIA-158V	
		TNP-4	TNP-26	TNP-4	TNP-26	TNP-4	TNP-26	TNP-4	TNP-26	TNP-4	TNP-26	TNP-4	TNP-26
IgG1	Untreated	++	++	+	++	++	+++	+	++	++	+++	++	+++
	EndoS	++	++	(+)	+	+	+++	(+)	+	–	–	+	++
	PNGase F	++	++	–	–	–	–	–	–	–	–	–	+
IgG2	Untreated	++	++	–	+	+	++	–	+	–	–	+	++
	EndoS	+	++	n.a.	(+)	–	++	n.a.	(+)	n.a.	n.a.	–	–
	PNGase F	–	+	n.a.	(+)	–	+	n.a.	(+)	n.a.	n.a.	–	–
IgG3	Untreated	++	++	+	+++	++	+++	+	++	++	+++	++	+++
	EndoS	++	++	(+)	+	+	+++	–	–	–	+	+	+++
	PNGase F	++	++	–	–	–	–	–	–	–	–	–	+
IgG4	Untreated	++	++	–	+	+	++	–	+	–	–	+	++
	EndoS	++	++	n.a.	(+)	–	–	n.a.	(+)	n.a.	n.a.	–	–
	PNGase F	+	+	n.a.	(+)	–	–	n.a.	(+)	n.a.	n.a.	–	–

The binding of EndoS- or PNGase F-treated TNP-specific IgG subclass ICs to the respective human FcγRs was graded as no binding (–), weak binding (+), good binding (++), and strong binding (+++).

n.a., Not applicable, because the respective IgG subclass does not bind to this receptor.

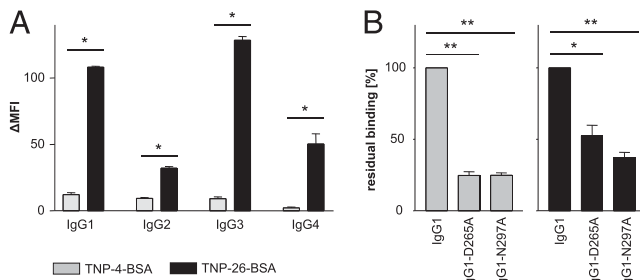


FIGURE 5. IC binding to human B cells. **(A)** Bar graphs indicate the binding of individual IgG subclasses complexed with TNP-4-BSA or TNP-26-BSA to the human B cell line LCL1.11. Data are mean fluorescence intensity (MFI) of three independent experiments $\pm$ SD. **(B)** Residual binding capacity of TNP-4-BSA and TNP-26-BSA in complex with human IgG1, IgG1-D265A, or IgG1-N297A. Bar graphs indicate the residual binding of TNP-specific IgG1-D265A and IgG1-N297A ICs to human B cells normalized to the binding of nonmutated TNP-specific IgG1 ICs (set to 100%) $\pm$ SD from three independent experiments. * p < 0.05, ** p < 0.01, Kruskal–Wallis test and subsequent post hoc tests.

CHO system, demonstrating that IgG1 and IgG3 ICs of the larger size show the best binding and that binding of IgG4 ICs is restricted to the larger complex. Moreover, binding of IgG1-D265A and IgG1-N297A variants was strongly reduced, although the size of the IC could partially overcome this block, especially for the D265A variant (Fig. 5B). We next turned our attention to cells of the innate immune system present in the peripheral blood. The majority of these cells (with the exception of NK cells) coexpress several activating Fc γ Rs, creating a rather complex scenario for IC binding. To address this experimentally, we incubated small and large ICs with PBMCs stained with Abs to detect neutrophils, NK cells, and inflammatory (CD33^{high}) and resident (CD33^{low}) monocyte subsets. Again confirming our data in the CHO model system, the large ICs bound better to the different innate immune cells (Fig. 6). To determine which Fc γ Rs contributed to the binding, we used either blocking Abs specific for Fc γ RI, Fc γ RIIA/IIIB, and Fc γ RIII or a combination of all Abs to completely block binding to Fc γ Rs, as done before (45). This analysis revealed that small and large IC binding to neutrophils and NK cells could be abrogated by preincubating the cells with the 3G8 Ab recognizing Fc γ RIIIA and Fc γ RIIIB, consistent with the high expression level of Fc γ RIIIB (CD16B) on neutrophils and Fc γ RIIIA (CD16A) on NK cells, as demonstrated before (Fig. 6A, 6B) (45). More interestingly, binding of small and large ICs to the CD33^{low} (resident) monocyte subset expressing Fc γ RIIA/B and Fc γ RIIIA was dependent on both of these receptors (Fig. 6C). In contrast, on inflammatory (CD33^{high}) monocytes expressing the high-affinity Fc γ RIA and Fc γ RIIA (but not Fc γ RIIIA), IC binding could be completely abrogated by blocking Fc γ RIIA (Fig. 6D). The minor contribution of Fc γ RIA to IC binding may be explained by the saturation of the high-affinity Fc γ RIA with serum IgG in the peripheral blood. Indeed, a much higher level of background staining is evident on this monocyte subset with the human IgG-specific secondary Ab that we used to detect bound ICs in the TNP-BSA control (Fig. 6D, 6H). Furthermore, we could detect a residual binding of EndoS-treated IgG1 ICs to Fc γ RIIIA/B on NK cells and neutrophil granulocytes. Because the blood donor was homozygous for the Fc γ RIIIA-158F allele, this is consistent with the results of the CHO-Fc γ RIIIA-158F cell line. With respect to Fc γ RIIA, our donor was heterozygous for the low-affinity Fc γ RIIA allele, which may explain why we were not able to demonstrate residual binding of EndoS-treated IgG1 ICs (Figs. 4A, 6G, 6H). Finally, we assessed whether the different sizes of ICs translate into different cellular responses. For this we incubated

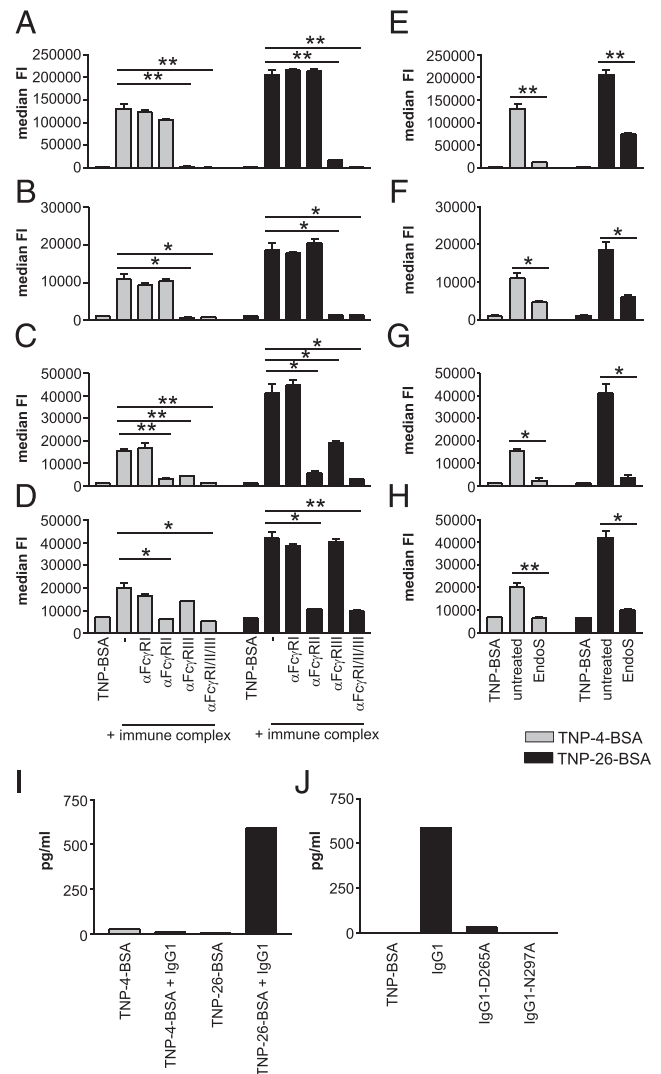


FIGURE 6. IC binding to human PBMCs. ICs were generated by coincubation of 10 μ g/ml of human anti-TNP IgG1 with 5 μ g/ml TNP-4-BSA or TNP-26-BSA, followed by incubation with human peripheral blood leukocytes. Cells were pretreated with the indicated Fc γ R-blocking Abs at 10 μ g/ml to analyze the influence of single Fc γ Rs on IC binding to neutrophils **(A)**, NK cells **(B)**, and CD33^{low} **(C)** and CD33^{high} **(D)** monocyte subsets. **(E–H)** ICs were generated with untreated or EndoS-digested TNP-specific IgG1 and incubated with human peripheral blood cells. As before, binding to neutrophils **(E)**, NK cells **(F)**, and CD33^{low}-expressing **(G)** and CD33^{high}-expressing **(H)** monocytes was analyzed by flow cytometry. Bar graphs indicate the median fluorescence intensity (FI) of one representative of three experiments performed in triplicate $\pm$ SD. **(I)** IL-6 secretion by whole human peripheral blood leukocytes after incubation with TNP-4-BSA or TNP-26-BSA ICs. **(J)** IL-6 secretion by whole human peripheral blood leukocytes after incubation of TNP-26-BSA ICs generated with anti-TNP IgG1, IgG1-D265A, or IgG1-N297A. One representative experiment of three is shown. * p < 0.05, ** p < 0.01, Kruskal–Wallis test and subsequent post hoc tests.

PBMCs with small and large IgG1 ICs and measured IL-6 secretion (Fig. 6I). Indeed, only the large IC was able to trigger IL-6 release. More interestingly, cytokine secretion was not blocked entirely by the IgG1-D265A variant, whereas no IL-6 secretion could be observed for the IgG1-N297A variant (Fig. 6J).

Discussion

The affinity of human IgG molecules for cellular Fc γ Rs ranges over several orders of magnitude. Thus, the high-affinity Fc γ RIA

is the only FcR with the capacity to bind monomeric IgG1, IgG3, and IgG4, whereas all of the other FcγRs interact solely with IgG in the form of multimeric ICs (5, 22, 36). Compared with the mouse system, human monomeric IgG subclasses have an even smaller affinity for the low-affinity FcRs FcγRIIA, FcγRIIB, and FcγRIIIA, making it difficult to obtain exact data using methods such as surface plasmon resonance. Moreover, it is unclear whether small differences in affinity and affinities below a certain threshold translate into functional binding to cellular FcγRs at all. Previous attempts to overcome this problem have either used heat-aggregated IgG or multimeric IgG generated by coinubation of IgG with anti-IgG F(ab')₂ fragments (22, 27). Although both approaches result in IgG aggregation, they may not reflect the interaction of IgG with its cognate Ag and do not allow for evaluation of the effect of different sizes of ICs on binding to cellular FcγRs. To resolve this issue, we used a set of TNP-specific human IgG1–4 subclasses and generated small and large ICs by coinubating these anti-TNP Abs with BSA carrying either 4 or 26 TNP groups. By studying the binding of these ICs to CHO cells expressing human FcγRs, we gained novel insights into human IgG IC interactions with cellular FcγRs. Thus, we identified human IgG2 ICs as a ligand of the high-affinity FcγRIA if present in the form of a small or large IC. More importantly, our study revealed that the size of an IC and individual FcγR alleles can strongly modulate this interaction, exemplified by the abundant binding of large IgG4 ICs to FcγRIIA, FcγRIIB, and FcγRIIIA and the allele-specific interaction of human IgG2 and IgG4 ICs with the FcγRIIIA-158V, but not the FcγRIIIA-158F, variant. This was unexpected, because the FcγRIIIA-158V allele (compared with the FcγRIIIA-158F allele) is largely considered to have a specific increase in binding to the IgG1 subclass. When comparing the binding pattern of small versus large ICs to human FcγRs, our data suggest that IgG1 and IgG3 have the highest capacity to interact with the low-affinity FcγRs even if present as small ICs, whereas IgG2 and IgG4 require a higher level of aggregation. Of note, these results emphasize that it is not possible to judge whether an IgG subclass binds to cellular FcγRs based on protein–protein interaction affinity data and that it seems to be quite difficult to determine a minimal affinity that is required to productively bind to cellular FcγRs. This may be exemplified by the IgG4-binding pattern observed in this study. Despite having a similar affinity of $2 \times 10^5 \text{ M}^{-1}$ for both alleles of the low-affinity FcγRIIIA, only the FcγRIIIA-158V allele could bind to this IC.

Perhaps more surprisingly, this study revealed that even the strongly reduced affinity of mutated IgG-Fc variants, such as the exchange of the aspartate residue at position 265 for an alanine residue in IgG1, selectively retains its capacity to bind to the FcγRIIA-131H allele if present in the form of a large IC. With respect to the high-affinity FcγRIA, our results emphasize that the high baseline affinity for IgG1 cannot be fully overcome by introduction of D265A or N297A mutations or by partially or fully deglycosylating IgG1 molecules; thus, it remains available to mediate IgG-dependent effector functions. Although this may not be of relevance for the blood, where the high-affinity FcγRIA may be saturated by high levels of serum IgG1 and IgG3 (as we also demonstrated in this study) or act only in combination with other activating FcγRs, this may still be of major importance for any tissue outside of the blood where FcγRIA is accessible for IC binding, as demonstrated in several mouse model systems in vivo (4, 46–51). In contrast to the small reduction in binding of EndoS- or PNGase F-treated IgG ICs to the high-affinity FcγR, a greater inhibition in binding was observed for the low-affinity FcγRs. Although this may be anticipated, several unexpected exceptions

became obvious. One striking example is the virtually normal binding of EndoS-treated large ICs of the IgG1, IgG2, and IgG3 subclasses to the FcγRIIA-131H allele and the fully intact binding of EndoS-treated IgG1 and IgG3 to the FcγRIIIA-158V allele.

By using human cell lines and primary cells of the peripheral blood, we were able to demonstrate that the results obtained in the CHO system seem to be matched by the binding data to human cells. More importantly, our data suggest that the size of the IC affects the resulting effector functions, as suggested by the higher amount of proinflammatory cytokines detected in the supernatant of PBMCs incubated with the large IC. Of note, our results should be interpreted with caution because there is evidence that FcγRs can have a cell type-specific glycosylation pattern. Thus, FcγRIIIA on human NK cells was suggested to contain more high-mannose and complex-type oligosaccharides than the same receptor on monocytes (52). Because no information about the different sugar structures attached to the various glycosylation sites in the individual FcγRs expressed on primary human cells is available, we can only speculate about the relevance of our data in this respect. Because the IC-binding patterns that we observed in the CHO system are largely recapitulated when using primary human cells, we speculate that the CHO-imposed glycosylation pattern may at least not result in major functional and structural differences. Indeed, despite some clear differences in the glycosylation pattern of FcγRIIIA produced either in CHO or HEK293 cells, there was only a mild difference in the affinity toward IgG1 (33). With respect to the physiological relevance of the size of the ICs used in this study, it should be noted that ICs present in the serum of patients with systemic lupus erythematosus compare with the larger complex (53). With respect to therapeutic Abs, it will be interesting to address the binding of cells coated with Abs to FcγR carrying effector cells. Finally, activation of the complement pathway and IC binding to complement receptors on innate immune effector cells also influence the binding pattern, which is not reflected in our current analysis (45). Indeed, there is convincing evidence that the epitope density of the Ag, Ab affinity, and, of course, the IgG subclass can have a strong effect on complement activation (54–57). Taken together, the results of this study emphasize that it is of the utmost importance to investigate the interaction of IgG with FcγRs in a cellular context and with IgG being in a state in which it is the physiological ligand (i.e., ICs) of these receptors.

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Disclosures

The authors have no financial conflicts of interest.

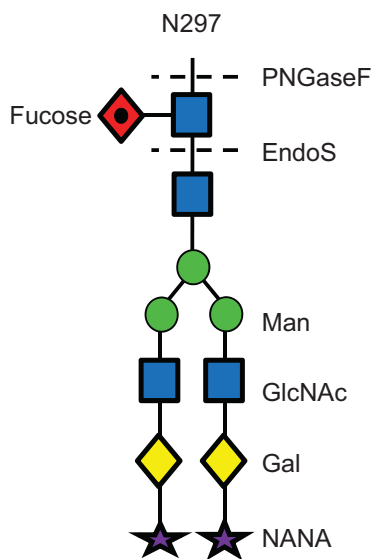
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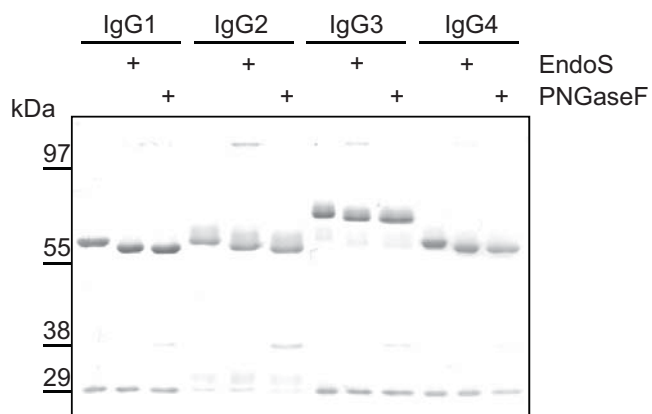
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Supplementary Figure 1 Immune complex size and Fcγ-receptors

A



B



C



Supplementary Figure 1: Generation of aglycosylated and partially deglycosylated human IgG subclasses.

(A) Shown is the branched structure of the asparagine 297 (N297) linked sugar moiety attached to the CH2 domain of human IgG and the respective sites for hydrolysis by EndoS and PNGaseF. Man: mannose, GlcNAc: n-acetylglucosamine, Gal: galactose, NANA: N-acetyl neuraminic acid. (B) TNP-specific human IgG subclasses were either left untreated or subjected to digestion with EndoS and PNGaseF. The removal of the mannose rich core sugar structure by these glycosidases was confirmed by lectin blotting using lens culinaris agglutinin (LCA). One out of three representative experiments is shown.