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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-acetylgalactosamine 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.

A
ntibodies 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 Fab 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-acetylgalactosamine, 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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A.L., C.N.S., and X.Y. performed experiments and analyzed the data; F.N., C.N.S., and A.L. wrote the manuscript and analyzed the data.

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Abbreviations used in this article: CHO, Chinese hamster ovary; EndoS, endoglycosidase S; IC, immune complex; PEG, polyethylene glycol; PNGase F, peptide; N-glycosidase F; TNP-26-BSA, BSA coupled to 26 2-,4-,6-trinitrophenyl groups; 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 large ICs.
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 FcγRI, FcγRIIB, FcγRIIA-131H, and FcγRIIB-
131H were generated in our laboratory by Anja Lux. CHO cell lines stably
expressing human FcγRIIA-158F and FcγRIIA-158V were kindly pro-
vided by M. Daeron (Institute Pasteur, Paris, France). Human FcγR ex-
pression on CHO cell lines was confirmed in each experiment by staining with
FITC-coupled Abs directed against FcγR (Jackson ImmunoResearch Labor-
atories). 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
binding to NK cells and myeloid cells, respectively. Binding to granulocytes was
detected by gating SSCneg 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 re-
combinant 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 (EndoV)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
benzotriolating, as previously described (26). IC binding to human peri-
pheral 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 in-
cubated 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 super-
natants were analyzed by cytokine bead array for the presence of IL-6
using the BD CBA Human Soluble Protein Flex Set Assay (BD Bio-
sciences), 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 FcγRIIA, the FcγRIIA-131H and FcγRIIA-131R allele, the
FcγRIIA-158F and FcγRIIA-158V 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 TNP-4–BSA coupled to either 4
TNP groups (TNP-4-BSA) or 2 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 gen-
eration 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
the 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 FcγRIIB variants. As shown in Fig. 1C, this identified a
very complex array of sugar structures attached to the various N-linked
 glycoproteins.
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 FcγRIIIA: only IgG3–TNP-26-BSA complexes showed a higher level of binding to FcγRIIIA-158F, and IgG4–TNP-26-BSA complexes demonstrated increased binding to FcγRIIIA-158V. Of note, the IgG4 subclass, usually considered to have low or no binding to FcγRIIA, FcγRIIB, and FcγRIIIA, 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 FcγRIIIA-158V allele, but not to the FcγRIIIA-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–Fc-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...
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 FcγRIIIA 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. 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. 3B). However, as for the D265A variant, only a minor effect of the N297A mutation on binding to the high-affinity FcγRIIA 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 FcγRIIA, 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 FcγRIIA. 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 FcγRIIA; however, it is sufficient to interact with FcγRIIA 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.
The binding of TNP-specific IgG1, IgG1-D265A, and IgG1-N297A ICs to human FcyRs was graded as no binding (−), weak binding (+), good binding (++), and strong binding (+++).

| Table I. Influence of IC size on human IgG1-D265A and IgG1-N297A binding to human FcyRs |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Ab Variant      | TNP-4           | TNP-26          | TNP-4           | TNP-26          | TNP-4           | TNP-26          | TNP-4           | TNP-26          | TNP-4           | TNP-26          |
| IgG1            | ++              | ++              | ++              | ++              | +++             | ++              | ++              | +++             | ++              | +++             |
| D265A           | ++              | ++              | ++              | ++              | +++             | ++              | ++              | +++             | ++              | +++             |
| N297A           | ++              | ++              | ++              | ++              | +++             | ++              | ++              | +++             | ++              | +++             |

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

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 FcyRs (26, 40–42). In contrast to PNGase F, EndoS treatment retains the penultimate N-acetylgalactosamine 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 FcyRs 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–FcyR 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 FcyRIIA for the small and large IC (Fig. 4). All IgG subclasses showed a diminished binding to the low-affinity receptors FcyRIIa-131R, FcyRIIB, and FcyRIIa-158F, even in the form of a large IC. In contrast, IC size dramatically influenced EndoS-treated IgG binding to FcyRIIa-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 FcyRIIA allele if present as a large IC (Table II). With respect to the FcyRIIa-131H 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 FcyRIIa-158F was abolished, independent of IC size. In contrast to previous data suggesting that the interaction of monomeric IgG2b with soluble human FcyRs is not impaired by EndoS treatment, our results clearly indicate that EndoS-treated IgG2 shows a reduced or diminished interaction with low-affinity FcyRs. Overall, these data indicate that the presence of a core N-acetylgalactosamine residue (retained after EndoS but not PNGase F digestion) provides a residual affinity for FcyR. 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–FcyR 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 FcyRs, is that...
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

<table>
<thead>
<tr>
<th>IgG Subclass</th>
<th>Glycosidase Treatment</th>
<th>FcγRIA</th>
<th>FcγRIA-131R</th>
<th>FcγRIA-131H</th>
<th>FcγRIIB</th>
<th>FcγRIIA-158F</th>
<th>FcγRIIA-158V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TNP-4</td>
<td>TNP-26</td>
<td>TNP-4</td>
<td>TNP-26</td>
<td>TNP-4</td>
<td>TNP-26</td>
</tr>
<tr>
<td>IgG1</td>
<td>Untreated</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>EndoS</td>
<td>++</td>
<td>++</td>
<td>(+)</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>PNGase F</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IgG2</td>
<td>Untreated</td>
<td>++</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>EndoS</td>
<td>+</td>
<td>++</td>
<td>n.a. (+)</td>
<td>–</td>
<td>n.a. (+)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>PNGase F</td>
<td>–</td>
<td>+</td>
<td>n.a. (+)</td>
<td>–</td>
<td>n.a. (+)</td>
<td>–</td>
</tr>
<tr>
<td>IgG3</td>
<td>Untreated</td>
<td>++</td>
<td>++</td>
<td>(+)</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>EndoS</td>
<td>++</td>
<td>++</td>
<td>(+)</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>PNGase F</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IgG4</td>
<td>Untreated</td>
<td>++</td>
<td>++</td>
<td>n.a. (+)</td>
<td>–</td>
<td>n.a. (+)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>EndoS</td>
<td>+</td>
<td>++</td>
<td>n.a. (+)</td>
<td>–</td>
<td>n.a. (+)</td>
<td>–</td>
</tr>
</tbody>
</table>

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.
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 FcγRs, 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 (CD33high) and resident (CD33low) 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 FcγRs contributed to the binding, we used either blocking Abs specific for FcγRI, FcγRIIA/IIIb, and FcγRIII or a combination of all Abs to completely block binding to FcγRs, 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 FcγRIIA and FcγRIIB, consistent with the high expression level of FcγRIIB (CD16B) on neutrophils and FcγRIIA (CD16A) on NK cells, as demonstrated before (Fig. 6A, 6B) (45). More interestingly, binding of small and large ICs to the CD33low (resident) monocyte subset expressing FcγRIIA/B and FcγRIIIA was dependent on both of these receptors (Fig. 6C). In contrast, on inflammatory (CD33high) monocytes expressing the high-affinity FcγRI A and FcγRIIB (but not FcγRIIA), IC binding could be completely abrogated by blocking FcγRIIA (Fig. 6D). The minor contribution of FcγRIIA to IC binding may be explained by the saturation of the high-affinity FcγRIIA 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-26-BSA control (Fig. 6D, 6H). Furthermore, we could detect a residual binding of EndoS-treated IgG1 ICs to FcγRIIA/B on NK cells and neutrophil granulocytes. Because the blood donor was homozygous for the FcγRIIA-158F allele, this is consistent with the results of the CHO-FcγRIIA-158F cell line. With respect to FcγRIIA, our donor was heterozygous for the low-affinity FcγRIIA 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 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 FcγRs ranges over several orders of magnitude. Thus, the high-affinity FcγRIA...
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 FcγRIIIA, 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 coinoculation 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 coincubating these anti-TNP Abs with BSA carrying either four 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 FcγRIIA 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 × 10^7 M^-1 for both alleles of the low-affinity FcγRIIA, 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 FcγRII-131H allele if present in the form of a large IC. With respect to the high-affinity FcγRIIA, 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 FcγRIIA 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 FcγRIIA 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 FcγRIIIA-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, FcγRIIA 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
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References


