CD16a with oligomannose-type N-glycans is the only “low-affinity” Fc γ receptor that binds the IgG crystallizable fragment with high affinity in vitro

Ganesh P. Subedi  
Iowa State University, gsubedi@iastate.edu

Adam W. Barb  
Iowa State University, abarb@iastate.edu

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Abstract
Fc γ receptors (FcγRs) bind circulating IgG (IgG1) at the surface of leukocytes. Antibodies clustered at the surface of a targeted particle trigger a protective immune response through activating FcγRs. Three recent reports indicate that the composition of the asparagine-linked carbohydrate chains (N-glycans) of FcγRIIIa/CD16a impacted IgG1-binding affinity. Here we determined how N-glycan composition affected the affinity of the “low-affinity” FcγRs for six homogeneous IgG1 Fc N-glycoforms (G0, G0F, G2, G2F, A2G2, and A2G2F). Surprisingly, CD16a with oligomannose N-glycans bound to IgG1 Fc (A2G2) with a \( K_D = 1.0 \pm 0.1 \) nM. This affinity represents a 51-fold increase over the affinity measured for CD16a with complex-type N-glycans (51 ± 8 nM) and is comparable with the affinity of FcγRI/CD64, the sole “high-affinity” FcγR. CD16a N-glycan composition accounted for increases in binding affinity for the other IgG1 Fc glycoforms tested (10–50-fold). This remarkable sensitivity could only be eliminated by preventing glycosylation at Asn\(^{162}\) with an Asn-to-Gln mutation; mutations at the four other N-glycosylation sites preserved tighter binding in the Man5 glycoform. None of the other low-affinity FcγRs showed more than a 3.1-fold increase upon modifying the receptor N-glycan composition, including CD16b, which differs from CD16a by only four amino acid residues. This result indicates that CD16a is unique among the low-affinity FcγRs, and modifying only the glycan composition of both the IgG1 Fc ligand and receptor provides a 400-fold range in affinities.

Disciplines
Biochemistry | Biophysics | Molecular Biology | Structural Biology

Comments
This research was originally published in the Journal of Biological Chemistry. Subedi, Ganesh P., and Adam W. Barb. "CD16a with oligomannose-type N-glycans is the only “low-affinity” Fc γ receptor that binds the IgG crystallizable fragment with high affinity in vitro." Journal of Biological Chemistry 293, no. 43 (2018): 16842-16850. © the Author(s). doi: 10.1074/jbc.RA118.004998.
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Received for publication, July 20, 2018, and in revised form, August 29, 2018 Published, Papers in Press, September 13, 2018, DOI 10.1074/jbc.RA118.004998

Ganesh P. Subedi and Adam W. Barb

From the Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology Iowa State University, Ames, Iowa 50011

Edited by Luke O'Neill

Fc γ receptors (FcγRs) bind circulating IgG (IgG1) at the surface of leukocytes. Antibodies clustered at the surface of a targeted particle trigger a protective immune response through activating FcγRs. Three recent reports indicate that the composition of the asparagine-linked carbohydrate chains (N-glycans) of FcγRIIa/CD16a impacted IgG1-binding affinity. Here we determined how N-glycan composition affected the affinity of the "low-affinity" FcγRs for six homogeneous IgG1 Fc N-glycoforms (G0, G0F, G2, A2G2, and A2G2F). Surprisingly, CD16a with oligomannose N-glycans bound to IgG1 Fc (A2G2) with a $K_D = 1.0 \pm 0.1$ M. This affinity represents a 51-fold increase over the affinity measured for CD16a with complex-type N-glycans (51 ± 8 nM) and is comparable with the affinity of FcRI/CD64, the sole "high-affinity" FcγR. CD16a N-glycan composition accounted for increases in binding affinity for the other IgG1 Fc glycoforms tested (10–50-fold). This remarkable sensitivity could only be eliminated by preventing glycosylation at Asn162 with an Asn-to-Gln mutation; mutations at the four other N-glycosylation sites preserved tighter binding in the Man5 glycoform. None of the other low-affinity FcγRs showed more than a 3.1-fold increase upon modifying the receptor N-glycan composition, including CD16b, which differs from CD16a by only four amino acid residues. This result indicates that CD16a is unique among the low-affinity FcγRs, and modifying only the glycan composition of both the IgG1 Fc ligand and receptor provides a 400-fold range in affinities.

Immune cells bind to IgG through six human Fc γ receptors (FcγRs) that can induce or suppress a protective but potentially damaging immune response. Although the proteins involved in this recognition are well-described, significant questions remain regarding how each receptor interacts with the enormous diversity of IgG molecules in the serum and how cells of each lineage modify the antibody-binding properties of their receptors (1). Five FcγRs promote immune activation, and one suppresses signaling (FcγRIIb/CD32b). These six receptors are differentially expressed on various leukocytes and are further subdivided into one high-affinity receptor (FcγRI/CD64) and five low-affinity receptors (FcγRIIa,b,c/CD32a,b,c; FcγRIIa,b/CD16a,b). FcγRs form a fundamental defense against disease and require productive engagement with the majority of therapeutic mAbs to achieve a therapeutic benefit (2, 3).

IgG antibodies bind FcγRs through the invariant crystallizable fragment (Fc). However, not all IgG antibodies exhibit the same cytotoxic potential. One variable is the location of the epitope on a given antigen; buried epitopes may lead toFc γRs sequestration and prevent productive engagement of surface-borne FcγRs. An additional well-known variable is the composition of the asparagine-linked carbohydrate (N-glycan) attached to Asn297 of the IgG1 Fc (Fig. 1) (4, 5). Each IgG molecule contains an Asn297 glycan on both heavy chains, and this modification is required to bind FcγRs (6–8). Furthermore, the composition of this N-glycan, resulting from the template-independent synthesis and glycan remodeling in the Golgi during protein expression, impacts affinity for various FcγRs. IgG1 Fc contains predominantly complex-type bi- and triantennary N-glycans with variable incorporation of fucose, galactose, and N-acetyllactosamine (9). IgG1 Fc glycoforms containing fucose bind with at least 4-fold weaker affinity to CD16a (10–13). Thus, many mAbs are glycoengineered to improve effector functions by preventing fucosylation, which is found on ~95% of circulating IgG1 (14, 15). The improvement in treatment efficacy by glycoengineered mAbs is likely due to increased affinity for CD16 (16, 17).

Recent reports indicate FcγR N-glycan composition likewise impacts antibody-binding affinity (18–20). In one study, CD16a with minimally processed oligomannose-type Man5 N-glycans bound to IgG1 Fc with 12-fold greater affinity than CD16a with highly processed complex-type N-glycans. The impact of this affinity increase was unclear because it is believed the majority of cell surface glycoproteins, including the FcγRs, display highly processed N-glycans similar to those found on serum glycoproteins. Indeed, a recent report indicated CD16b isolated from human serum contained primarily highly processed complex-type N-glycans, with minimally processed forms found at the Asn45 glycosylation site (21). However,
CD16a from primary human natural killer cells contained a large amount of minimally processed N-glycans, which included a significant fraction of hybrid and oligomannose N-glycans (45%) (20, 22).

From these reports it was clear that a wide range of N-glycan species may be found decorating the low-affinity FcγRs expressed at the surface of human immune cells; however, it was not clear whether the function of each receptor would be expressed at the surface of human immune cells; however, it was not clear whether the function of each receptor would be affected by N-glycan composition as much as CD16a. Furthermore, the magnitude to which N-glycan composition impacted CD16a remains undefined because only a limited panel of IgG1 Fc glycoforms were used in the prior study. The goal of the experiments described here is to determine the extent to which receptor N-glycan composition impacted affinity for IgG1 Fc in vitro by testing a range of different receptors and Fc glycoforms. We likewise sought to determine whether the glycan at one specific site of the receptor, of the many found on the heavily glycosylated FcγRs, contributes a greater degree toward the receptor sensitivity to N-glycan composition.

Results
Preparation and N-glycan analysis of recombinant Fcγ receptors

Transiently transfected human embryonic kidney (HEK) 293S cells expressed the soluble, antibody-binding extracellular domains of the low-affinity FcγRs (rCD16a, rCD16b, rCD32a, and rCD32b) to high yields and high purity following elution from a nickel–nitrilotriacetic acid column (80–120 mg liter−1; Fig. S1). The HEK293S (GntI−) cell line synthesizes N-glycoproteins with primarily Man5 N-glycans because of a GNTI gene deletion that prevents the later stages of N-glycan processing (23). LC-MS–based analysis of these recombinant receptors revealed the presence of >99% oligomannose-type N-glycans (Fig. 2). We previously reported the binding affinity of each low-affinity FcγR, expressed in the parent HEK293F cell line, to an array of IgG1 Fc N-glycoforms (12). A parallel analysis of these HEK293F-expressed receptors indicated that >89% of the glycans were of a complex type (CT) (Fig. 2B).

Binding-affinity measurements

Each of the four low-affinity Fcγ receptors with Man5 N-glycans bound to IgG1 Fc glycovariants immobilized on a surface. These experiments tested binding to six homogeneous IgG1 Fc glycovariants: G0, G0F, G2, G2F, A2G2, and A2G2F with G0 indicating zero galactose residues on the complex-type biantennary N-glycan branches, A2 indicating the presence of two N-acetyleneuraminic acid residues on the branches, and F indicating a single core fucose residue. Once binding reached a kinetic equilibrium, the intensity at equilibrium provided a measure of the amount of receptor bound to the single covalently anchored IgG1 Fc glycoform. Fitting a binding isotherm to these equilibrium intensity values provided an estimate for the dissociation constant and is shown with representative data in Figs. 3 and 4. The complete data are compiled in Table 1 and Table S1.

The general patterns of relative affinity among the low-affinity Fcγ receptors with oligomannose-type N-glycans proved comparable with those measured with complex-type N-glycans. rCD16a bound the tightest, followed by rCD32a, rCD32b, and rCD16b when using fucosylated IgG1 Fc. The patterns between receptors with complex-type and oligomannose N-glycans were likewise similar when binding to afucosylated IgG1 Fc. In this case, rCD16a bound the tightest, followed by rCD32a, rCD32b, and rCD16b in descending order of affinity. Thus, both rCD16a-Man5 and rCD16b-Man5 but neither rCD32a-Man5 nor rCD32b-Man5 exhibited a marked sensitivity to IgG1 Fc fucosylation. This relative binding-affinity ranking is consistent with previous results observed using the complex-type glycoforms (12).

Of the receptors analyzed, only rCD16a with Man5 oligomannose N-glycans showed a dramatic increase in affinity when compared with rCD16a with complex-type N-glycans. rCD16a-Man5 bound to afucosylated IgG1 Fc (G0, G2, and
Figure 2. *N*-Glycan composition of the recombinant Fc γ receptors. Elution pattern and glycan composition of *N*-glycans from recombinant Fc γ receptors using hydrophilic interacting chromatography–LC/MS/MS are shown. A–D, expression of receptors using HEK293F cells provides primarily CT *N*-glycans (A and B) and Man5 *N*-glycans (C and D) results from expression using HEK293S (Gnt1-) cells. The numbers in the pie charts indicate the percentages of the *N*-glycan groups.
A2G2) with 41–51-fold greater affinity than rCD16a-CT, compared with an increase of 0.7–3.1-fold for rCD16b, rCD32a, and rCD32b (Table 1). The binding affinity to fucosylated IgG1 Fc glycoforms (G0F, G2F, and A2G2F) increased between 10- and 16-fold, compared with a 1.2–2.4-fold increase for rCD16b, rCD32a, and rCD32b.

The affinity of rCD16a-Man5 for afucosylated IgG1 Fc (A2G2) is >400-fold tighter than the affinity of rCD16a-CT.
binding IgG1 Fc (G0F). This astonishing result indicates that an enormous range of binding affinities are achievable by modifying only the N-glycan composition of the ligand and receptor.

Furthermore, the $1.0 \pm 0.1$ nM affinity measured for the IgG1 Fc (A2G2)–rCD16a-Man5 interaction is comparable with the 1–3 nM binding affinity of the high-affinity Fcγ receptor CD64 (11,

Figure 4. Representative binding analysis for fucosylated IgG1-Fc (G0) measured by SPR. The left column shows binding sensograms, and the right column shows fits using response intensity values once a binding equilibrium is reached. Error bars for the binding fits are shown.
Thus, rCD16a is unique among the low-affinity Fcγ receptors with an unprecedented sensitivity to N-glycan composition and the capability to bind afucosylated IgG1 Fc in vitro with affinities comparable to CD64. It is surprising that this unique sensitivity is not shared by rCD16b, which differs by only four amino acid residues in the extracellular antibody-binding domains analyzed (Fig. S2).

The role of individual CD16a N-glycans

Previous reports indicated that two of the five potential CD16a N-glycans contribute to IgG1 Fc-binding affinity (19, 25–27). Indeed, glycans at Asn45 and Asn162 proved essential for the high-affinity interactions described above. Removing the Asn38, Asn74, and Asn169 glycosylation sites through mutation to Gln slightly increased the affinity (Fig. 5 and Table S1). However, removing N-glycosylation sites by mutating either Asn45 or Asn162 mostly reduced affinity. Although the N45Q variant with complex-type N-glycans showed weaker affinity for IgG1 Fc than the N162Q variant, the relative positions were reversed when measuring the affinity of the Man5 receptor glycoforms. Surprisingly, binding affinity measured for the rCD16a-N45Q variant still showed sensitivity to the receptor glycoform: the Man5 glycoform bound more than 10-fold tighter to IgG1 Fc than the CT glycoform. This sensitivity was lost with the rCD16a-N162 variant, which showed comparable binding in either glycoform to IgG1 Fc. Thus, the rCD16a Asn162 glycan mediates high-affinity interactions with IgG1 Fc. This result can be explained by the location of the Asn162 glycan at the interface formed by IgG1 Fc and rCD16 observed in high-resolution structures determined by X-ray crystallography (Fig. S3), although there is considerable disagreement related to the nature of the interactions at this site (19, 28–31).

The role of receptor fucosylation

Multiple laboratories previously demonstrated that fucosylation of the IgG1 Fc core (1)GlcNAc residue reduced the affinity for rCD16a from 4- to 50-fold (10, 12). One major difference between rCD16a-Man5 and rCD16a-CT is that the former glycoform is not fucosylated. We assessed the role of receptor fucosylation by comparing the binding of rCD16a displaying complex-type glycans expressed with or without fucose to determine whether the increased affinity of rCD16a-Man5 could be explained by the missing fucose residue (+fuc or −fuc, respectively). The impact of receptor fucosylation proved minimal for three rCD16a amino acid variants analyzed, with most interactions perturbed by less than a 2-fold change in affinity as compared with a 10–51-fold change upon replacing complex-type N-glycans with Man5 glycans (Fig. 6 and Table S1). One exception to this conclusion is that rCD16a-CT(−fuc) binds IgG1 Fc G0 with 5-fold greater affinity than rCD16a-CT(+fuc),

Table 1

| Receptor | N-glycan composition impacts binding affinity | IgG1 Fc G0F | IgG1 Fc G0 |
|----------|---------------------------------------------|-------------|-------------|
| rCD16a-Man5 | 25 | 25 | 2.5 | 1.0 |
| rCD16b-Man5 | 3300 | 3300 | 590 | 80 |
| rCD16b-Man5 | 720 | 720 | 870 | 80 |
| rCD16b-Man5 | 3000 | 3000 | 500 | 50 |
| rCD16a-CT | 409 | 32 | 101 | 12 |
| rCD16b-CT | 6250 | 300 | 757 | 21 |
| rCD32a-CT | 1220 | 110 | 1370 | 70 |
| rCD32b-CT | 3740 | 260 | 1980 | 200 |

Table 1. Receptor and IgG1 Fc N-glycan composition impacts binding affinity

| Receptor | N-glycan processing (CT/Man5) |
|----------|-----------------------------|
| rCD16a | 16.4 |
| rCD16b | 1.9 |
| rCD32a | 1.7 |
| rCD32b | 1.2 |

* CT values from Ref. 12.

Figure 5. rCD16a amino acid substitutions and N-glycan composition affects IgG1 Fc binding affinity. Errors of fit for the dissociation constants are shown.

Figure 6. The effect of receptor fucosylation on binding affinity. rCD16a was expressed in the absence (CT + fuc) or presence (CT − fuc) of 2-deoxy-2-fluoro-l-fucose. Error bars represent the error of the dissociation constant fit from equilibrium intensity data.
but this affinity increase is still far below the 40-fold increase upon comparing rCD16a-CT and rCD16a-Man5 binding IgG1 Fc G0. Thus, it is unlikely that the predominant contribution to the increased affinity of rCD16a-Man5 for IgG1 Fc can be explained by a lack of CD16a fucose.

Discussion

These experiments demonstrate CD16a is uniquely sensitive to the composition of its covalently attached N-glycans, as well as the ligand N-glycans in vitro. Of the five possible CD16a N-glycans, Asn^162 appears to be primarily responsible for this behavior, and the Asn^162 glycan was previously implicated as the primary moiety sensing antibody fucosylation (30, 31). It is remarkable that protein function can be modified by N-glycan composition; however, IgG1 Fc N-glycan composition is a well-described modifier of CD16a binding (5, 6, 12). The surprising aspects of these findings are the magnitude of the modulation and that the sensitivity to N-glycan composition is restricted to a single low-affinity FcγR.

Modifying the biantennary complex-type IgG1 Fc N-glycan with fucose, galactose, and N-acetyleneuraminic acid generates an 8-fold range of affinities for rCD16a (12). Modifying receptor N-glycan composition impacts rCD16a-binding affinity by up to 51-fold. These effects combined provide a 400-fold range of affinities for rCD16a, with the predominant impact from receptor N-glycan composition. This wide range of affinities potentially provides the body an opportunity to tune the immune system by modifying N-glycans. A significant amount of evidence indicates IgG1 Fc N-glycans change in response to multiple stressors, including age and disease (including but not limited to Refs. 32–38), but there are very few reports of FcγR glycosylation in native human tissues because of the difficulty in obtaining sufficient material for analysis (20, 21).

It would be appropriate to assume that the low-affinity FcγRs, which share a high degree of structural and sequence homology, share similar functional profiles; however, rCD16a is by far the most sensitive to the composition of its attached N-glycans with up to a 51-fold change in binding affinity, but the highly related rCD16b is only impacted by a 3.1-fold change. This is even more surprising considering that rCD16b shares a similar sensitivity to IgG1 Fc N-glycan composition with an observed 16-fold range of affinities as compared with 8-fold for rCD16a, 1.8-fold for rCD32a, and 2.2-fold for rCD32b (12).

One weakness of this present study is related to the reductionist approach measuring monovalent interactions of the soluble extracellular domains with the IgG1 Fc in vitro. It is unknown how these observed changes in monovalent binding affinity impact the multivalent interactions that hold an opsonized target to the surface of an immune cell; it is likely that the raw affinities measured in this study do not accurately recapitulate affinities for similar interactions in the complex milieu of the serum or peripheral tissues that contain a multitude of factors including 5–15 mg/ml competing antibody and occur at a membrane. However, we expect that relative differences between the receptors we identified are representative of the in vivo activity. It is noteworthy that the modest 4-fold affinity enhancement achieved by preventing antibody fucosylation or the 5-fold increase in affinity observed for the CD16a-V158 v. CD16a-F158 allotype increases patient outcome with therapeutic monoclonal antibodies (11, 39–42). Thus, if modest changes in receptor–antibody interactions provide measurable advantages in patient outcome, then the potential to exploit a larger 51-fold enhancement is noteworthy.

This study highlights the critical need to determine the N-glycan composition of FcγRs from primary, uncultured human tissue to develop recombinant systems that produce comparable material for in vitro binding studies. Although substantial resources have been previously applied to analyze N-glycans from recombinant receptors (43–45), it is evident that even human cell-based recombinant protein expression systems do not appropriately recapitulate receptor glycosylation because CD16a from primary human natural cells and HEK293F expression are dramatically different (20). CD16a isolated from natural killer cells donated by three older male donors contained smaller complex type N-glycans plus a significant proportion of hybrid and oligomannose types, unlike rCD16a from HEK293F or serum-borne CD16b that contained predominantly large complex-type N-glycans and a small amount of oligomannose forms (20, 21). Therefore, based on these results, the rCD16a used to assess antibody binding imperfectly represents the native CD16a glycoforms and is an inaccurate model of antibody-binding affinities on immune cell surfaces.

Engineering antibody N-glycan composition is proving to be a powerful device to enhance the effector properties of therapeutic monoclonal antibodies (16, 17, 46, 47). Although it is unclear whether the human body modifies antibody N-glycan composition to directly tune sensitivity of the immune system or whether antibody glycosylation changes result from change in the immune system, the clinical evidence supporting the enhanced efficacy of glycoengineered antibodies is substantial. Changes in antibody glycosylation have a much smaller impact on receptor-binding affinity than changes in the receptor glycans and implicate receptor glycosylation as an unexplored device to enhance drug efficacy.

Experimental procedures

Materials

Materials were purchased from Sigma–Aldrich unless otherwise noted.

Protein expression and purification

A description of the preparation, purification, in vitro remodeling, and analysis of the batch of human IgG1 Fc (residues 216–447) glycovariants used in this study was published previously because these glycovariants were previously used for binding analyses (12). The low-affinity Fc γ receptors with complex-type N-glycans including recombinant (r)CD16a and associated variants (residues 19–193, Val^158 allotype), rCD16b (residues 19–193), rCD32a (residues 43–216, LR (H143) allotype), and rCD32b (residues 43–216) were expressed with HEK293F cells as previously described (12, 48, 49). rCD16a and related variants were also expressed with HEK293F cells in the presence of 250 μM 2-deoxy-2-fluoro-l-fucose (Santa Cruz Biotechnology) to prevent fucosylation (50). Receptors with Man5 oligomannose N-glycans were expressed using HEK293S
(GntI-1) (51). N-Glycans from the low-affinity Fc γ receptors were released, purified, conjugated to procoaminide, and analyzed using hydrophilic interacting chromatography–MS on a Q-Exactive mass spectrometer (ThermoFisher) as described previously (20). The spectra were analyzed using Byonic (Protein Metrics) to identify singly, doubly, or triply charged N-glycan species. Each identification was manually validated by analyzing retention time and MS2 spectra using Xcaliber (Thermo Fisher).

**Binding-affinity measurements**

Fc was coupled onto a CM5 sensor chip on a Biacore T100 instrument (GE Life Sciences). Fc γ receptors were flowed over the Fc-coupled chip as previously described (12). A minimum of two experiments for each receptor/Fc pair was collected on at least two different days, and representative data are reported. Dissociation constants for each experiment were determined by fitting the equilibrium response values at each receptor concentration to the Hill equation. All binding experiments using WT FcγRs with oligomannose-type N-glycans were collected at the same time as the previously reported binding affinity measurements for the WT FcγRs with complex-type N-glycans and are thus directly comparable (12).

**Author contributions—G. P. S. and A. W. B. conceptualization; G. P. S. and A. W. B. data curation; G. P. S. and A. W. B. formal analysis; G. P. S. and A. W. B. investigation; G. P. S. and A. W. B. visualization; G. P. S. and A. W. B. methodology; G. P. S. and A. W. B. writing—original draft; G. P. S. and A. W. B. writing—review and editing; A. W. B. supervision; A. W. B. funding acquisition; A. W. B. project administration.

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CD16a with oligomannose-type N-glycans is the only "low-affinity" Fc \( \gamma \) receptor that binds the IgG crystallizable fragment with high affinity \textit{in vitro}.

Ganesh P. Subedi and Adam W. Barb

\textit{J. Biol. Chem.} 2018, 293:16842-16850.  
doi: 10.1074/jbc.RA118.004998 originally published online September 13, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA118.004998

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