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Effects of anchor structure and glycosylation of Fcγ receptor III on ligand binding affinity

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ABSTRACT Isoforms of the Fcγ receptor III (FcγRIII or CD16) are cell surface receptors for the Fc portion of IgG and important regulators of humoral immune responses. Different ligand binding kinetics of FcγRIII isoforms are obtained in three dimensions by surface plasmon resonance and in two dimensions by a micropipette adhesion frequency assay. We show that the anchor structure of CD16 isoforms isolated from the cell membrane affects their binding affinities in a ligand-specific manner. Changing the receptor anchor structure from full to partial to none decreases the ligand binding affinity for human IgG1 (hIgG1) but increases it for murine IgG2a (mlgG2a). Removing N-glycosylation from the CD16 protein core by tunicamycin also increases the ligand binding affinity. Molecular dynamics simulations indicate that deglycosylation at Asn-163 of CD16 removes the steric hindrance for the CD16-hIgG1 Fc binding and thus increases the binding affinity. These results highlight an unexpected sensitivity of ligand binding to the receptor anchor structure and glycosylation and suggest their respective roles in controlling allosterically the conformation of the ligand binding pocket of CD16.

INTRODUCTION

In humans, type III cell surface receptors for the Fc portion of immunoglobulin (Ig) G (Fcγ receptor III or CD16) are encoded by two genes, A and B, which give two protein products, CD16a and CD16b, respectively (Kimberly et al., 2002; Nimmerjahn and Ravetch, 2008). CD16a is expressed on macrophages, mast cells,
CD16b\textsuperscript{NA1}-transfected cells with tunicamycin (an inhibitor of N-glycosylation) results in increased binding of monomeric IgG, whereas the same treatment of cells transfected with N163Q mutant of CD16b\textsuperscript{NA1} has no effect. These results suggest that glycosylation at Asn-163, which is shared by all three CD16 membrane isoforms and located in the ligand binding pocket (Sondermann et al., 2000; Radaev et al., 2001; Ferrara et al., 2011), regulates the affinity of CD16 for IgG (Drescher et al., 2003). However, the extent to which the glycosylation affects the ligand binding affinity and kinetics has not been quantified. The structural basis for the glycosylation effects also has not been elucidated.

The cocystal structures of CD16b\textsuperscript{NA2} and CD16a in complex with an Fc fragment of human IgG1 (hFc1) have been independently solved by several groups (Sondermann et al., 2000; Radaev et al., 2001; Ferrara et al., 2011). Although the crystals obtained have different space group symmetries, the structures are almost identical, showing a 1:1 stoichiometry for binding. CD16 adopts the characteristic heart-shaped domain arrangement as described for soluble Fc\textgamma RIIb and other soluble FcRs. Compared with the standalone structure, CD16 further opens its interdomain angle by 10° when it is liganded with hFc1. Concurrently, hFc1 opens asymmetrically upon complex formation, upon which both CH2 domains bend away from the C2 axis of the homodimeric hFc1, but one CH2 domain is dislocated more than the other.

Ligand binding kinetics of CD16 has been studied using surface plasma resonance (SPR; Galon et al., 1997; Maenaka et al., 2001; Li et al., 2007). In SPR, one binding partner is immobilized onto a sensor surface over which the other binding partner flows in a fluidic phase to interact in three dimensions. Soluble aglycosylated CD16b\textsuperscript{NA2} produced by Escherichia coli used in two studies yielded drastically different kinetic rates (Galon et al., 1997; Maenaka et al., 2001), despite the fact that the respective materials from the two groups produced the same crystal structure (Sondermann et al., 2000; Radaev et al., 2001). One group also compared glycosylated with aglycosylated CD16b\textsuperscript{NA2} and showed that they have similar binding affinity for human IgG1 (hIgG1; Galon et al., 1997). The similarity in three-dimensional (3D) affinity is at odds with the observation that the glycosylation of CD16 greatly affects its ligand binding (Edberg and Kimberly, 1997; Drescher et al., 2003).

Ligand binding of CD16 triggers signaling and effector functions such as phagocytosis and antibody-dependent cellular cytotoxicity (Nagarajan et al., 1995b). Cell surface CD16 binds small immune complex in three dimensions and IgG-opsonized surfaces in two dimensions (Nagarajan et al., 1995b). Ligand binding kinetics of CD16 has also been measured in two dimensions by micropipette adhesion assay, which analyzes receptor–ligand interactions across the junctional gap between two apposing surfaces (Chesla et al., 1998, 2000; Williams et al., 2000a,b, 2001; Shashidharanmurthy et al., 2009). This assay can examine effects of membrane organization and cellular environment of the interacting molecules that cannot be addressed by SPR. The differences between two-dimensional (2D) and 3D binding have been highlighted by studies of binding of T-cell receptors (TCRs) and coreceptors with peptide-major histocompatibility complex (pMHC) molecules (Huang et al., 2010; Huppa et al., 2010; Adams et al., 2011; Jiang et al., 2011; Sabatino et al., 2011). Indeed, the micropipette assay shows that, compared with transmembrane (TM) CD16a (CD16a\textsuperscript{TM}), CD16a\textsuperscript{gPI} binds faster and with higher affinity to human and rabbit IgGs but slower and with lower affinity to murine IgG2a (mlIgG2a; Chesla et al., 2000). Previous study suggests that the membrane anchor influences ligand binding by conformational change of CD16 (Chesla et al., 2000).

In the study, we further explored the effect of anchor structure of CD16 on its ligand binding using three different methods to generate soluble CD16s with different anchor structures, allowing us to isolate the effect of linker segment from the membrane anchor itself on the intrinsic binding parameters. We also tested how glycosylation of the receptor affected ligand binding, by using tunicamycin treatment to block the addition of sugar moieties to the protein core. Our results show that affinity of CD16 binding to hIgG1 correlates with receptor anchor structures, and this correlation is inverted upon switching ligand from hIgG1 to mIgG2a, suggesting that long-range conformational change on the receptor anchor could propagate to the ligand binding epitope. Aglycosylated CD16 has higher affinity for hIgG1 than the glycosylated forms, suggesting an important role of posttranslational modification in regulating receptor binding affinity. Molecular dynamics (MD) simulations show that deglycosylation of CD16 removes the steric hindrance for CD16–hIgG1 binding, resulting in increased binding affinity.

RESULTS

Solubilized CD16 captured on microspheres specifically binds IgG coated on red blood cells

To test for binding specificity, adhesion frequencies of IgG coated on red blood cells (RBCs) to solubilized CD16 of different forms and anchor structures (Figure 1) captured by 214.1 precoated on microspheres were compared with several controls. Adhesion frequencies were measured with a 2-s contact duration. As exemplified using microspheres incubated with various CD16 lysates (Figure 2A), the ~50% adhesion frequencies to hIgG1-coated RBCs were reduced to ~10% by the anti-CD16 antibody, 3G8. Furthermore, use of microspheres incubated with bovine serum albumin (BSA) instead of CD16a\textsuperscript{TM} lysate or RBCs not coated with anything (but undergoing
The kinetic information is embedded in the curves of adhesion frequency versus contact duration, as exemplified by the data in Figure 2B, measured with CD16aTM lysate–coated microspheres and CD16aGPI lysate–coated microspheres incubated with hIgG1-coated RBCs. The higher specific adhesion frequency for total human IgG than CHO cell CD16aTM (Chesla et al., 2000). This result was confirmed by a new set of micropipette experiments (Figure 3A). Here we measured binding frequencies at multiple contact durations and then fitted the data to Eq. 2 to obtain affinities and off-rates as shown in Figure 2B. We showed that the differential 2D affinities observed previously using CHO cell CD16aGPI lysate and CD16aTM lysate had comparable 2D off-rates for dissociation from hIgG1 (Figure 3B), consistent with previous findings for cell-surface CD16a membrane isoforms (Chesla et al., 2000).

The findings that hIgG1 bound with a higher 2D affinity to CD16aGPI expressed on CHO cells had a higher 2D affinity for total human IgG than CHO cell CD16aTM (Chesla et al., 2000). This result was confirmed by a new set of micropipette experiments (Figure 3A). Here we measured binding frequencies at multiple contact durations and then fitted the data to Eq. 2 to obtain affinities and off-rates as shown in Figure 2B. We showed that the differential 2D affinities observed previously using CHO cell CD16aGPI lysate and CD16aTM lysate had comparable 2D off-rates for dissociation from hIgG1 (Figure 3B), consistent with previous findings for cell-surface CD16a membrane isoforms (Chesla et al., 2000).

CD16 anchor structure affects 2D binding affinity for hlgG1

The findings that hlgG1 bound with a higher 2D affinity to CD16aGPI than CD16aTM even after the receptors were captured from the cell lysates on the microspheres (Figure 3A) exclude differential diffusivities, flexibilities, heights, orientations, and organizations on the membrane, as well as any other cell-associated factors between the two isoforms, as possible causes for the affinity difference. We therefore focused on the structural differences in the membrane anchor per se. We hypothesized that the membrane anchor affects ligand binding by inducing conformational changes in the ligand binding site (Chesla et al., 2000). Because the membrane anchor is one Ig domain away from the ligand binding site, the proposed anchor effect on binding affinity must be exerted allosterically. Perturbing the structure along this allosteric pathway of regulation is therefore predicted to alter the binding affinity.

To test this hypothesis, we used lysed, phosphatidylinositol-specific phospholipase C (PIPLC) treatment, and spontaneous shedding to obtain different anchor structures of solubilized receptors from CD16aGPI or CD16aNA2-expressing CHO cells. Lysing the cells keeps the molecule’s original GPI anchor. PIPLC treatment enzymatically cleaves the diacylglycerol moiety of the GPI anchor. Note that PIPLC does not affect N-glycans on CD16. Spontaneous shedding results from the action of metalloproteinases bound to the

the same CrCl3 procedure except adding IgG (Chesla et al., 2000). Reduced the adhesion frequency to the background level (≤2%).

The kinetic information is embedded in the curves of adhesion frequency versus contact duration, as exemplified by the data in Figure 2B, measured with CD16aTM lysate–coated microspheres and hlgG1-coated RBCs using seven contact durations, t, from 0.5 to 16 s. Five microsphere–RBC pairs were tested at each contact duration, and 100 contacts were repeated for each pair to calculate a total adhesion frequency, P1 (number of adhesions observed divided by the 100 contacts). Nonspecific adhesion frequency, P2, was controlled using the 214.1-coated microspheres incubated with lysates of plain CHO cells (Figure 2B). The specific adhesion frequency P3 was then calculated using the following equation (Williams et al., 2001):

\[
P_3 = \frac{(P_1 - P_2)}{1 - P_2}
\]

The P3 versus t binding curve was fitted by Eq. 2 together with the separately measured receptor and ligand densities (m and m, respectively; Chesla et al., 1998):

\[
P_3 = 1 - \exp(-m_mA_K[A_c(1 - \exp(-k_{off}t)])
\]

to estimate K, and k_{off}, the 2D affinity (in μm²) and off-rate (in s⁻¹), respectively. The 2D on-rate can be calculated from K,m = K,k_{off}. The K value is lumped with the contact area A_c in the curve fit, which is called collectively the effective 2D affinity. We kept A_c constant in all experiments.

CD16aGPI lysate binds hlgG1 with a higher affinity than CD16aTM lysate

Previously we showed that CD16aGPI expressed on CHO cells had a higher 2D affinity for total human IgG than CHO cell CD16aTM (Chesla et al., 2000). This result was confirmed by a new set of micropipette experiments (Figure 3A). Here we measured binding frequencies at multiple contact durations and then fitted the data to Eq. 2 to obtain affinities and off-rates as shown in Figure 2B. We showed that the differential 2D affinities observed previously using CHO cell CD16aGPI lysate and CD16aTM lysate had comparable 2D off-rates for dissociation from hlgG1 (Figure 3B), consistent with previous findings for cell-surface CD16a membrane isoforms (Chesla et al., 2000).

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cell membrane, which produces molecules with only the extracellular domain (Lanier et al., 1989). The micropipette adhesion assay was applied to measure 2D affinities between hlgG1 and these molecules at an 8-s contact duration, long enough for the interaction to reach equilibrium (Figure 2B). Specific adhesion frequencies, $P_a$, were obtained by removing background adhesion frequencies from total adhesion frequencies using Eq. 1 and then substituting into the following equation to calculate the effective 2D affinities:

$$A_K = \frac{1}{K_d} = -\ln(1 - P_a) / m$$

This equation is a transformation of the equilibrium (i.e., $t \to \infty$) version of Eq. 2.

Changing the anchor structure of CD16aGRIP from full to partial to none resulted in a progressive decrease in its affinity for hlgG1 (Figure 4A, middle). The same result was seen with CD16bNA2, also a GPI-anchored molecule (Figure 4A, right). Further confirmation was obtained using CD16aTM, as the molecule with the full anchor structure plus cytoplasmic tail (lysate) showed a much higher affinity for hlgG1 than the molecule with no anchor (shedding; Figure 4A left). The PIPLC treatment was not used because PIPLC cannot cleave the polypeptide transmembrane anchor of CD16aTM. Student’s $t$ test confirmed that the affinity differences within each group of CD16 were significant ($p$ values in Figure 4A). These results support our hypothesis regarding allosteric regulation of ligand binding affinity by receptor anchor.

The anchor effect on ligand binding is inverted when hlgG1 is replaced by mlgG2a (Figure 4). Comparisons of 2D affinities of hlgG1 (A) and mlgG2a (B) for CD16. CD16 molecules are captured on 214.1-precoated microspheres from CHO cell lysates, supernatants of CHO cells treated with PIPLC, or supernatants of CHO cells subjected to shedding treatment. Adhesion frequencies were measured with an 8-s contact duration and converted to 2D effective affinities using Eq. 3.

The anchor effect on ligand binding is inverted when hlgG1 is replaced by mlgG2a (Figure 4). Comparisons of 2D affinities of hlgG1 (A) and mlgG2a (B) for CD16. CD16 molecules are captured on 214.1-precoated microspheres from CHO cell lysates, supernatants of CHO cells treated with PIPLC, or supernatants of CHO cells subjected to shedding treatment. Adhesion frequencies were measured with an 8-s contact duration and converted to 2D effective affinities using Eq. 3. Data are mean $\pm$ SEM of 5–15 RBC–microsphere pairs with 100 contacts each per bar. Data values are below the plots; $p$ values from Student’s $t$ test are above data bars.

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\[ A_K = -\ln(1 - P_a) / m \]
GPI anchor had intermediate affinity (Figure 4B). Again, Student's t test confirmed the significance of the differences in 2D affinities (p values in Figure 4B).

Glycosylation of CD16 molecules affects their ligand binding affinities

The effect of the receptor anchor structure on the ligand binding affinity may be changed by perturbations in the structure of the ligand binding site, as it resides on the other end of the proposed allosteric pathway of binding affinity regulation. One possible structure is the glycan attached to Asn-163 of CD16, which is inside the binding pocket of the CD16–hFc1 complex (Sondermann et al., 2000; Radaev et al., 2001; Ferrara et al., 2011). We thus prepared deglycosylated CHO cell CD16 molecules by adding tunicamycin, a deglycosylated CHO cell CD16 molecules (Figure 5B).

Deglycosylation of CD16 enhances CD16–hFc1 binding

To understand the structural basis for the effects of CD16 glycosylation on its ligand binding, we performed all-atom, explicit-solvent MD simulations for both glycosylated and aglycosylated CD16a liganded with hFc1, which contains two glycans on Asn-297. The starting structure of the simulation of the glycosylated CD16a-hFc1 complex was a crystal structure (Protein Data Bank 3SGJ; Ferrara et al., 2011) in which two N-glycans are attached to Asn-46 and Asn-163. The starting structure of the aglycosylated CD16a-hFc1 complex was obtained by removing in silico the glycans of CD16a in the crystal structure. After 10 ns in the simulations, the root mean square deviations of the Cα atoms of the complexes reached a plateau, suggesting that the systems were equilibrated (Supplemental Figure S1). The time between the aglycosylated and glycosylated complexes is 4.5 Å. Then the contact time is calculated as the fraction of the total time that the two residues are in contact. The difference in contact time that the two residues are in contact. The difference in contact time between the aglycosylated and glycosylated complexes is shown in Figure 6. Positive or negative value indicates that the interaction is strengthened or weakened upon deglycosylation, respectively. Some interactions between the N-terminal loops of hFc1 and α residues of CD16a were not present in the starting structure of the glycosylated CD16a-hFc1 complex. To exclude the possibility that the increase of effective 2D affinities of CD16 for hlgG1 with tunicamycin treatment was due to a cellular effect, we measured 2D binding of a soluble CD16a–Ig chimera for hlgG1. In agreement with the results of CHO cell CD16, the effective 2D binding affinity of hlgG1 was fourfold higher for CD16a–Ig secreted from CHO cells with tunicamycin treatment than without (Figure 5A, far right), confirming that the affinity increases in the cell-surface CD16 for hlgG1 were due to the change in the receptor molecules. The two-orders-of-magnitude higher effective 2D affinities of hlgG1 for soluble CD16a–Ig than for CHO cell-surface CD16a, regardless of whether they were for glycosylated or deglycosylated molecules, are consistent with the increase by one to two orders of magnitude in effective 2D affinities of hlgG1 for CD16a lysates on microspheres (Figure 3A); both can be explained by the increases in effective contact area, A. This is consistent with our previous report that CD16bNA2 reconstituted on RBCs has a ∼50-fold-higher effective 2D affinity for hlgG than the same CD16bNA2 reconstituted or transfected on CHO cells (Williams et al., 2001).

Finally, we measured 3D affinities of an anti-CD16 monoclonal antibody (mAb; CLBFcgran-1) for different CD16 isoforms using saturation binding experiments and Scatchard plot analysis (Figure 5C). Consistently, deglycosylation also increased the 3D affinity of CLBFcgran-1 for CD16a (Figure 5D). These results support our hypothesis that glycosylation perturbs the structure of CD16.
CD16 are weakened (blue spheres in Figure 6C). However, more interactions between the CH2 domain of hFc1 and CD16 are strengthened (red spheres in Figure 6C). Of interest, the strengthenerations of CD16 affects the ligand binding affinity even in an acelluler system. We also demonstrated that glycosylation provides highest affinity for mlgG2a (Figure 4). The observation further suggests that the anchor effect is caused by CD16 itself rather than its cellular environment. To dissect the ligand specificity of the anchor effect, we compared the amino acid sequences of hIgG1 and mlgG2a (Supplemental Figure S2). The two sequences are highly conserved, with >60% identity. However, in the regions directly interacting with mlgG2a: CD16 with full membrane anchor has highest affinity for hIgG1 but lowest affinity for mlgG2a. The observation further supports that the anchor effect is caused by CD16 itself rather than its cellular environment. To dissect the ligand specificity of the anchor effect, we compared the amino acid sequences of hIgG1 and mlgG2a (Supplemental Figure S2). The two sequences are highly conserved, with >60% identity. However, in the regions directly interacting with mlgG2a: CD16 with full membrane anchor has highest affinity for hIgG1 but lowest affinity for mlgG2a. This change in anchor structure increases the negative charge of IgG and thus may strengthen the interaction with Lys-132 of CD16 (Supplemental Figure S3). His-268 and Glu-269 are conserved anchor effects.

Another important new finding is the preservation of the ligand specificity of the anchor effect, such that the changes of affinity of CD16 for hIgG1 with changes of anchor structure are opposite to that for mlgG2a: CD16 with full membrane anchor has highest affinity for hIgG1 but lowest affinity for mlgG2a, whereas CD16 without anchor has the lowest affinity for hIgG1 but the other side of the ligand binding site, still affects ligand binding affinity (Figure 4). This allows us to rule out the differential diffusivities, flexibilities, heights, orientations, and organizations on the membrane, as well as any other cell-associated factors of these molecules, as possible causes for the observed anchor effects.

Another important new finding is the preservation of the ligand specificity of the anchor effect, such that the changes of affinity of CD16 for hIgG1 with changes of anchor structure are opposite to that for mlgG2a: CD16 with full membrane anchor has highest affinity for hIgG1 but lowest affinity for mlgG2a, whereas CD16 without anchor has the lowest affinity for hIgG1 but the
shedding form used in our micropipette experiments. We speculate that the binding interface is changed through an allosteric mechanism when CD16 has an anchor, and the change depends on the anchor form. For the PIPLC-cleaved form of CD16, the interacting residues between CD16 and IgG may all be conserved between hlgG1 and mlgG2a, so that their affinities for CD16 are similar. However, for CD16 with a full anchor, other, nonconserved residues may interact with CD16, and these interactions are favored for hlgG1, so that hlgG1 has higher affinity for CD16 than mlgG2a.

Although our findings may seem surprising, many examples of allosteric regulation of ligand binding have been reported. In interactions between selectins and ligands (Lou et al., 2006; Lou and Zhu, 2007), between platelet glycoprotein Ibα and von Willebrand factor (Yago et al., 2008), and between bacterial FimH receptor and mannoside ligand (Le Trong et al., 2010), force-induced conformational changes may give rise to catch bonds (Dembo et al., 1988; Marshall et al., 2003). In integrins, allosteric conformational changes are related to inside-out and outside-in signaling (Luo et al., 2007) and may cause catch bonds (Kong et al., 2009; Chen et al., 2010, 2012; Xiang et al., 2011). Closer to our findings, the GPI-anchored protein Thy-1 has been shown to lose reactivity with several mAbs and a polyclonal antibody after anchor cleavage by phospholipase C, indicating that the GPI anchor affects the protein conformation (Barboni et al., 1995; Kukulansky et al., 1999).

Notwithstanding the foregoing examples, that truncating the membrane anchor of a receptor may affect its ligand binding has been underappreciated. It is a common practice to make recombinant soluble receptors with membrane anchors truncated and replaced by different tags for purification by affinity chromatography. Almost all structural determinations of extracellular domains of cell surface receptors by crystallography and nuclear magnetic resonance use molecules made in this way. Molecules so made are also widely used for kinetics measurements by SPR (Galon et al., 1997; Maenaka et al., 2001; Li et al., 2007). It is an implicit but usually untested assumption that the soluble receptors have identical structures and ligand binding properties as the native molecules on the cell membrane. This assumption has been challenged by work re-calculating the difference in 2D affinity of the CD16-IgG binding has a large effect on the binding of CD16 to immune complexes. In vivo, the change of the CD16–IgG binding affinity by posttranslational modification of CD16 such as glycosylation may be a way to regulate immune responses. In fact, CD16 encoded by the same gene could undergo differential cell type–specific glycosylation (Edberg and Kimberly, 1997; Drescher et al., 2003), and so the CD16-expressing cells may be selectively activated in an immune response. The importance of posttranslational processes is also observed in other molecular systems, such as CD8, for which nonsialylated glycoforms are present in immature thymocytes but virtually absent in mature thymocytes. This glycosylation difference is linked to the difference in ligand binding affinity between CD8 on mature and immature thymocytes (Moody et al., 2003).

Our MD simulations suggest that deglycosylation at Asn-163 of CD16 removes the steric hindrance for the CD16–FcR binding and therefore enhances the binding affinity (Figure 6). This provides a structural explanation for the glycosylation effects on the CD16–IgG binding shown by us (Figure 5) and others (Drescher et al., 2003).

In summary, we explored the effect of anchor structure and glycosylation of CD16 on ligand binding using three different methods to solubilize CD16 molecules with distinct anchor forms. The results extended our previous study (Chesla et al., 2000) and showed that the membrane anchor structure itself and glycosylation can regulate allosterically these intrinsic binding parameters.

**MATERIALS AND METHODS**

**Cells and antibodies**

Transfected CHO cells expressing human CD16a™, CD16αGPI, and CD16bN42, as well as untransfected control CHO cells, were cultured...
as described previously (Chesla et al., 1998, 2000). The expressions of various forms of CD16s were periodically checked with flow cytometry. The anti-CD16 nonblocking mAb 214.1 (murine IgG1; Fleit et al., 1992) was a generous gift from Howard Fleit (State University of New York, Stony Brook, NY). The anti-CD16 adhesion blockade mAb CLBFCgran-1 and 3G8 were purified from hybridomas (Selvaraj et al., 1988). HlgG1 was kindly provided by Adrian Whitty (Biogen, Boston, MA) and was purchased from Sigma-Aldrich (St. Louis, MO). Fluorescein isothiocyanate–labeled goat anti-human Fc-specific and goat anti-mouse Fc-specific antibodies (Sigma-Aldrich) and phycoerythrin (PE)-labeled goat anti-human Fc-specific and goat anti-mouse Fc-specific antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used to estimate the site density of hlgG1, CLBFCgran-1, and mlgG2a. The mouse anti-human IgG Fc mAb 7QD was purchased from General Bioscience (Brisbane, CA).

**Preparation of solubilized CD16 and soluble CD16a–Ig chimera**

One gram of each CD16-expressing CHO cell pellet was resuspended in lysis buffer containing 1% Triton X-100 and protease inhibitors and incubated for 30 min at 4°C (Nagarajan et al., 1995a). Protease inhibitors included the serine protease inhibitors phenylmethylsulfonyl fluoride (1 mM) and aprotinin (5 mg/ml) and the cysteine protease inhibitor iodoacetamide (1 mM). The lysate was clarified by centrifugation at 100,000 × g for 1 h at 4°C. Supernatant was collected and stored at −80°C for further use. For CD16b and CD16a(G1), 1% octyl glucoside was added to lyse the cells and preserve the GPI anchor. To prepare PIPLC cleavage of GPI-anchored CD16, 107 cells were incubated in 1 ml phosphate-buffered saline (PBS)/5 mM EDTA, pH 7.4, with 0.2 unit of PIPLC for 1 h at 37°C. To prepare shed CD16, 106 CD16-expressing CHO cells were incubated in 1 ml of Hanks balanced salt solution at 37°C for 3 h with gentle mixing. The CD16 molecules so generated are schematically shown in Figure 1 in different forms as labeled by lysate, PIPLC, and shedding. CD16a–Ig chimera was generated as described (Li et al., 2002) and is also depicted in Figure 1. Briefly, the chimeric CD16a–Ig cDNA was transfected into CHO cells, which were then cultured using CHO serum-free medium. The culture supernatant was collected, and CD16a-Ig was purified using a Sepharose protein G column by affinity chromatography.

**Generation of CHO cells expressing aglycosylated CD16**

Tunicamycin blocks N-glycosylation synthesis by inhibiting the transfer of N-acetylgalcosamine-1-phosphate to dolicholmonophosphate. At high concentrations, tunicamycin treatment drastically reduces the expression level of CD16. Twofold serial dilution of tunicamycin was performed to determine the optimal concentration (78 ng/ml) that blocks the glycosylation of CD16 and yet does not significantly alter its expression level. CHO cells transfected to express cell-surface CD16 or CD16a-Ig were rinsed three times using culture medium containing 78 ng/ml tunicamycin (Sigma-Aldrich) and cultured in the same medium for 40 h at 37°C. Cells expressing CD16 membrane isoforms were then rinsed three times with clean culture medium, detached, and analyzed by FACS for CD16 expression or by micropipette for IgG binding. Supernatant from the CD16a-Ig–expressing cell culture was collected, and CD16a–Ig was purified using Sepharose protein G column by affinity chromatography.

**Coupling of capturing antibody to microspheres**

After being washed twice in carbonate buffer, 10 million carboxylated microspheres were washed three times in phosphate buffer (pH 4.5) and resuspended in 120 μl of phosphate buffer. The same volume of 2% carbodiimide solution was added dropwise to the microsphere suspension, and the mixture was incubated for 3–4 h at room temperature with agitation. Unreacted carbodiimide was removed by three washes with phosphate buffer and resuspended in 200 μl of borate buffer. Next 20–40 μg of 214.1 or 7QD was added to the microsphere mixture and incubated overnight at room temperature with mixing. Microspheres were collected, and the antibody concentration in the supernatant was tested by the Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) to estimate the efficiency of coupling. The pellet was resuspended in 0.2 M borate buffer, and 50 μl of 0.1 M methanolamine was added and mixed gently for 30 min at room temperature to block unreacted sites on the microspheres. Microspheres were then incubated in 10 mg/ml BSA solution for 30 min at room temperature. Finally, microspheres were collected and stored in PBS with 1% BSA at 4°C for future use.

**Chromium chloride coupling of antibody to RBCs**

HlgG1, mlgG2a, or F(ab')2 of mAb 7QD was covalently coupled to the membranes of RBCs using a previously described CrCl3 method (Chesla et al., 1998). A 1% CrCl3 solution was prepared, properly aged at pH 5, and diluted in 20 mM acetate-buffered saline, pH 5.5, at ratios ranging from 1:6700 to 1:17,000. Fresh RBCs were washed five times in saline and resuspended to 2% hematocrit. IgG was added to each 250-μl sample and mixed. An equal volume of diluted CrCl3 solution was added dropwise with constant agitation. After 5 min, the reaction was stopped by addition of 0.5 ml PBS/5 mM EDTA/1% BSA. Cells were subsequently washed and stored in EAS45 as described previously (Dumaswala et al., 1996). An aliquot from each sample was examined under light microscopy for aggregation. Samples were assayed for coating density and uniformity by flow cytometry.

**Site-density determination**

Site densities of proteins on RBCs (CrCl3-coupled IgG or soluble CD16–Ig captured by precoated 7QD) and microspheres (solubilized CD16 captured by precoated 214.1) were determined by quantitative fluorescence immunosassay (Chesla et al., 1998, 2000; Williams et al., 2000a,b, 2001; Shashidharamurthy et al., 2009). Samples were prepared for flow cytometry as described (Chesla et al., 2000). The mean fluorescence intensities of the RBCs or microspheres were compared with standard calibration beads (Bangs Laboratories, Fishers, IN, and Becton Dickinson, San Jose, CA) to determine the mean number of fluorophores per cell or microsphere, which was then converted into labeled protein per cell.

**Micropipette adhesion frequency assay**

Two-dimensional affinities and kinetic rates of CD16 for IgG were measured by the micropipette adhesion frequency assay as previously described (Chesla et al., 1998). Briefly, 103 CHO cells expressing membrane CD16, microspheres captured with solubilized CD16, or RBCs captured with CD16–Ig and 105 ligand-coated RBCs were respectively added to separate corners of a cell chamber sufficient far apart to avoid mixing. The cell chamber was filled with RPMI (Mediatech, Manassas, VA) plus 1% BSA, 5 mM EDTA, and 0.04% sodium azide and mounted on the stage of an inverted microscope (Axiovert 100; Zeiss, Oberkochen, Germany). A single CD16-bearing CHO cell, microsphere, or RBC and a single IgG-coated RBC were respectively aspirated by two apposing micropipettes and aligned via micromanipulation. One pipette was mounted to a
computer-driven piezoelectric translator to move the two cells into contact for a predetermined area and duration. On pipette retraction, the two cells were either immediately separated (i.e., no adhesion, scored 0) or remained bound, which stretched the RBC for a short time before it was detached by force (i.e., adhesion, scored 1). This adhesion test cycle was repeated 100 times to estimate adhesion frequency, and three or five pairs of cells were used to obtain a mean and SEM of adhesion frequency for each contact duration, which was varied from 0.5 to 16 s.

**Antibody saturation binding experiment**

The affinities of the anti-CD16 mAb CLBFcgran-1 for CD16 isoforms expressed on CHO cells were measured by saturation binding with twofold serial dilution. CLBFcgran-1 at varying concentrations was incubated with CD16-expressing cells for 30 min in triplicate wells containing FACS buffer. Cells were then washed and stained with PE-conjugated goat anti-mouse Fc specific antibody for an additional 30 min. Cells were washed, and the expression of CD16 was checked via flow cytometry. Controls were performed both with no staining and with secondary antibody staining alone without CLBFcgran-1.

**Molecular dynamics binding experiment**

The starting structure for the glycosylated CD16-hFc1 complex was the published cocrystal structure downloaded from the Protein Data Bank (PDB code 3SGJ; Ferrara et al., 2011). The starting structure for the aglycosylated CD16-hFc1 complex was obtained by removing in silico the glycans on CD16 in the crystal structure. The simulation systems were prepared using LEaP in AmberTools14 (Case et al., 2014). The Amber ff12SB (Maier et al., 2015) and GLYCAM06 (Kirschner et al., 2008) force fields were used for proteins and glycans, respectively. Each starting structure was placed into a truncated octahedral periodic box of TIP3P water molecules with a 15-Å minimal distance between the edges of the water box and the solute. Sodium and chloride ions were added to neutralize the systems and produce a 150 mM ionic strength. MD simulations were performed using NAMD (Phillips et al., 2005). The particle mesh Ewald method (Darden et al., 1999) was used to treat long-range electrostatic interactions. The cutoff for non-bonded interactions was set to 12 Å, with a switching function starting from 10 Å. SHAKE (Ryckaert et al., 1977) was used to constrain lengths of bonds involving hydrogen. Time-step was 2 fs. The systems were energy minimized using the conjugate gradient method in two steps: first with heavy atoms of the proteins fixed, and then with heavy atoms of the proteins harmonically restrained (force constants of 5 kcal/mol Å²). After minimization, the systems were gradually heated from 0 to 310 K during 120-ps canonical ensemble (NVT)-MD simulations with harmonic restraints (force constants of 5 kcal/mol Å²) on heavy atoms of the proteins. Subsequent isothermal isobaric ensemble (NPT)-MD simulations were performed for 5 ns with the same harmonic restraints. Then the harmonic restraints were reduced to zero in four steps with respective force constants of 1, 0.1, 0.01, and 0 kcal/mol Å². Each step contains a 1-ns NPT-MD simulation. Finally, NPT-MD production simulations were performed for 50 ns each. The simulations were run on XSEDE (Towns et al., 2014).

VMD (Humphrey et al., 1996) and PyMOL (version 1.7) were used for visualization and analysis. Two residues were defined as in contact if any pair of their heavy atoms of side chains (Cα atom for glycine) were within 4.5 Å. Then the contact time was calculated as fraction of total time the two residues were in contact. Only the last 40 ns was used to calculate contact time.

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**Anchor and glycosylation effects of CD16**


