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A Perspective on the Structure and Receptor Binding Properties of Immunoglobulin G Fc

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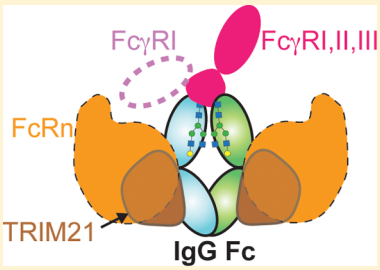
ABSTRACT: Recombinant antibodies spurred a revolution in medicine that saw the introduction of powerful therapeutics for treating a wide range of diseases, from cancers to autoimmune disorders and transplant rejection, with more applications looming on the horizon. Many of these therapeutic monoclonal antibodies (mAbs) are based on human immunoglobulin G1 (IgG1) or contain at least a portion of the molecule. Most mAbs require interactions with cell surface receptors for efficacy, including the Fc γ receptors. High-resolution structural models of antibodies and antibody fragments have been available for nearly 40 years; however, a thorough description of the structural features that determine the affinity with which antibodies interact with human receptors has not been published. In this review, we will cover the relevant history of IgG-related literature and how recent developments have changed our view of critical antibody−cell interactions at the atomic level with a nod to outstanding questions in the field and future prospects.



I. INTRODUCTION

Immunoglobulin G1 (IgG1) is a dual-function molecule. Interactions between the fragment antigen binding (Fab) and target antigens are of high affinity, developed through clonal selection and an affinity maturation process that optimizes the amino acid sequence of the variable regions of the IgG heavy and light chains. In the case of an invading pathogen, multivalent pathogen-specific IgGs will coat the surface of the pathogen (opsonization) through Fab regions that recognize surface antigens. This process clusters and orients the fragment crystallizable (Fc) region of IgG to interact with cell surface receptors, including the Fc γ receptors (FcγRs). The IgG Fc receptor family is comprised of one high-affinity receptor (nanomolar affinity), FcγRI, and several low-affinity receptors (micromolar affinity), FcγRIIa, FcγRIIb, and FcγRIIIa.[1−4] Fc elicits antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Fc is also capable of eliciting intracellular antibody-mediated degradation in a wide variety of cell types.[5] This process is triggered when antibody-coated virions enter the cytoplasm and are recognized by the cytosolic Fc binding protein TRIM21.

IgG1 is a heterotetramer that consists of two "heavy" polypeptide chains and two "light" chains (Figure 1). The heterotetramer structure is covalently stabilized through disulfide bonds that link both heavy chains together in the hinge regions and link each "light" chain to a "heavy" chain. Fab and Fc both retain their individual functions after proteolytic separation; thus, structure- and activity-based investigation of the individual IgG components provides information about the entire molecule. Fc is released as a 52 kDa homodimer following papain digestion of the IgG1 molecule. Each monomer contains a Cγ2 domain and a Cγ3 domain (Figure 1). The Cγ3 domains of each monomer interact to form a strong noncovalent dimer interface. The Cγ2 domain is the site of many receptor interactions and contains a conserved asparagine 297-linked (N-linked) carbohydrate chain (glycan). This is a structural feature of note as the Fc N-glycan is required for interactions with receptors such as FcγRIIIa and FcγRIIa, but not FcγRI, TRIM21, or the neonatal Fc receptor (FcRn).

IgG1 Fc is a popular target for studies of protein structure determined by X-ray crystallography, and high-resolution models have been available for nearly 40 years.[6−26] Structural studies also revealed how Fc interacts with a wide variety of receptors through a diverse set of interfaces.[9−11,16,17,22,27] An interesting feature of all these models is the near complete resolution of the conserved Fc N-glycan.[12−14,21,24] However, these models do not indicate why the N-glycan is necessary for proper IgG1 and mAb function. Preliminary studies indicate the behavior of the N-glycan is much more complex than these models reveal,[28,29] and a hypothesis linking N-glycan structure with Fc receptor binding activity remains elusive.

It is clear that an approach integrating high-resolution structural methods and measurements of Fc affinity in solution will be required to thoroughly evaluate the Fc structure−activity relationship. Here we will present the results and interpretations of studies of human Fc using a wide range of solution and solid-state methods as well as in vitro measurements of interactions of Fc with human receptors.

II. STRUCTURAL ASPECTS OF IGG FC

IIa. Cγ2 Domain Orientations. Domain orientations are a well-characterized feature of many Fc models. The C-terminal

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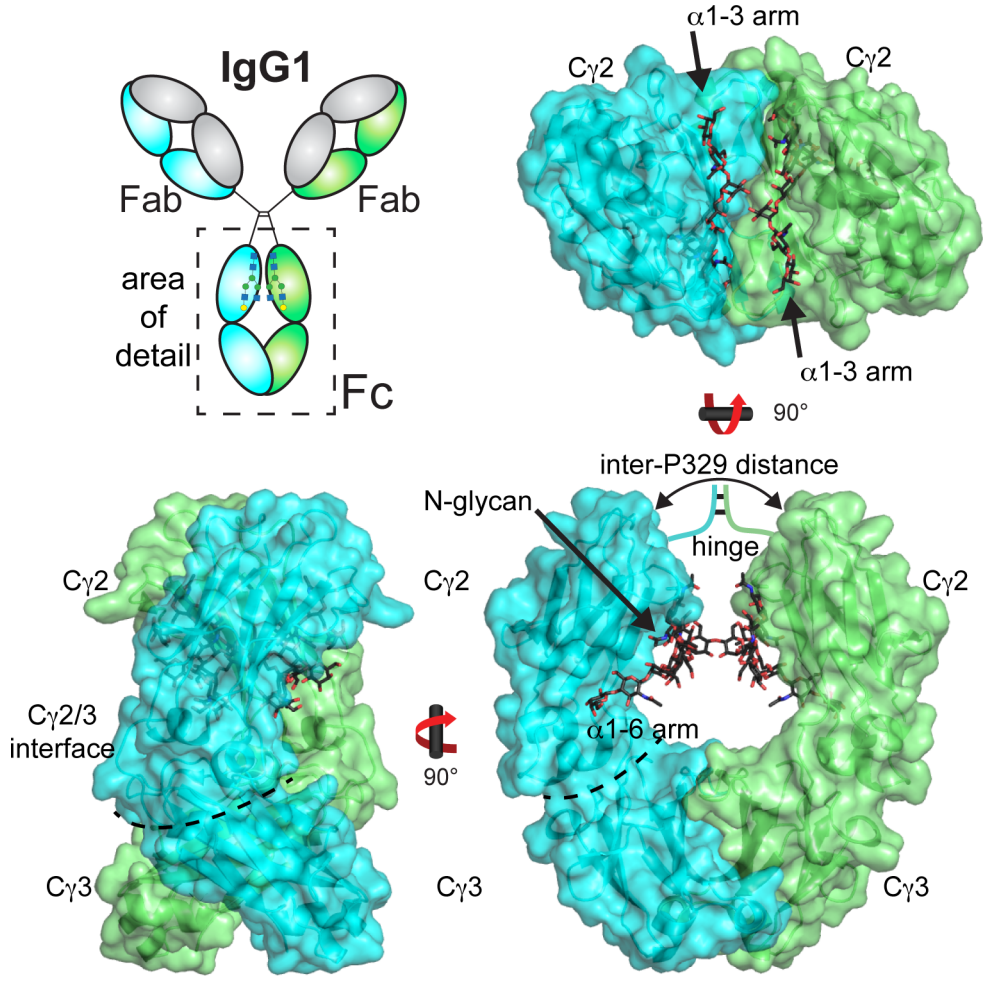

Figure 1. Structure of IgG1 Fc (Protein Data Bank entry 1L6X). Chain A of the homodimer is colored cyan and chain B green.

half of the "heavy" polypeptide chain forms the IgG1 Fc homodimer (residues 225−447).[30] Though symmetric in solution with a 2-fold rotational symmetry axis formed by a dimer interface,[31] Fc rarely crystallizes in a symmetric pose. Deviations from a symmetric structure are largely limited to the positions of the Cγ2 domains; the dimer interface formed by the Cγ3 domains appears to be structurally invariant. Differences in Cγ2 domain orientation suggest the Cγ2 domains are not rigid with respect to one another. This may be important for determining the role of Fc motions, particularly the Cγ2 domains, in receptor binding because Fc binds Fc γ receptors I−III via the lower hinge region between Cγ2 domains (Figure 2). Furthermore, FcγRI−III all form an interface with both Fc Cγ2 domains; thus, Cγ2 motion and relative domain orientation are thought to influence the Fc−FcγR interaction.[15]

Several different parameters have been used to compare relative Fc domain orientations. These measurements provide a useful frame of reference for comparing Fc structures as determined by X-ray crystallography, but the biological relevance of these structural deviations is unclear. One commonly reported measurement is the distance between P329 residues.[20,21,24] P329 is located in the FG loop of the Cγ2 domain (Figures 1 and 2). Inter-P329 distances describe the distance between Cγ2 domains in Fc. The shortest reported P329 distance is 18.9 Å in an aglycosylated Fc structure.[24] This observation led to the hypothesis that aglycosylated Fc assumes a collapsed structure and it was inferred that the N-glycan serves to prevent this collapsed conformation from forming and maintaining the FcγR binding properties. However, an unpublished aglycosylated Fc structure [Protein Data Bank (PDB) 3DNK] has a P329 distance of 27.6 Å that is very similar to those of many glycosylated structures (27.4 Å for PDB entry 4KU1[8]). It is currently unclear which observed Fc conformation more accurately reflects the likely ensemble of solution conformations. Extensive molecular dynamics (MD) simulations indicate the Cγ2 domains access a degree of motion (75−108°) significantly larger than that described by X-ray crystallography (91−104°).[8]

Fc conformation is a complex property, and it is unclear what range of conformations Fc samples and what effect this distribution has on receptor binding. Attempts to more fully describe Fc conformation include additional distance measurements (P238, F241, R301, and the C1 atom of Man4[20,21]) or the definition of interdomain angles. Descriptions of simple three-point angles formed by the Cγ2 and Cγ3 domains[8,9] or a four-point dihedral angle between Cγ2 and Cγ3 domains[8] allow description of the degree of Fc asymmetry. Regardless of the mode by which asymmetry is measured, it is clear from the multiple poses observed by X-ray crystallography and molecular dynamics simulations, the Cγ2 domains are mobile and the relative orientation in space is not required to be symmetric.[8] The role of this conformational heterogeneity in Fc function remains undefined.

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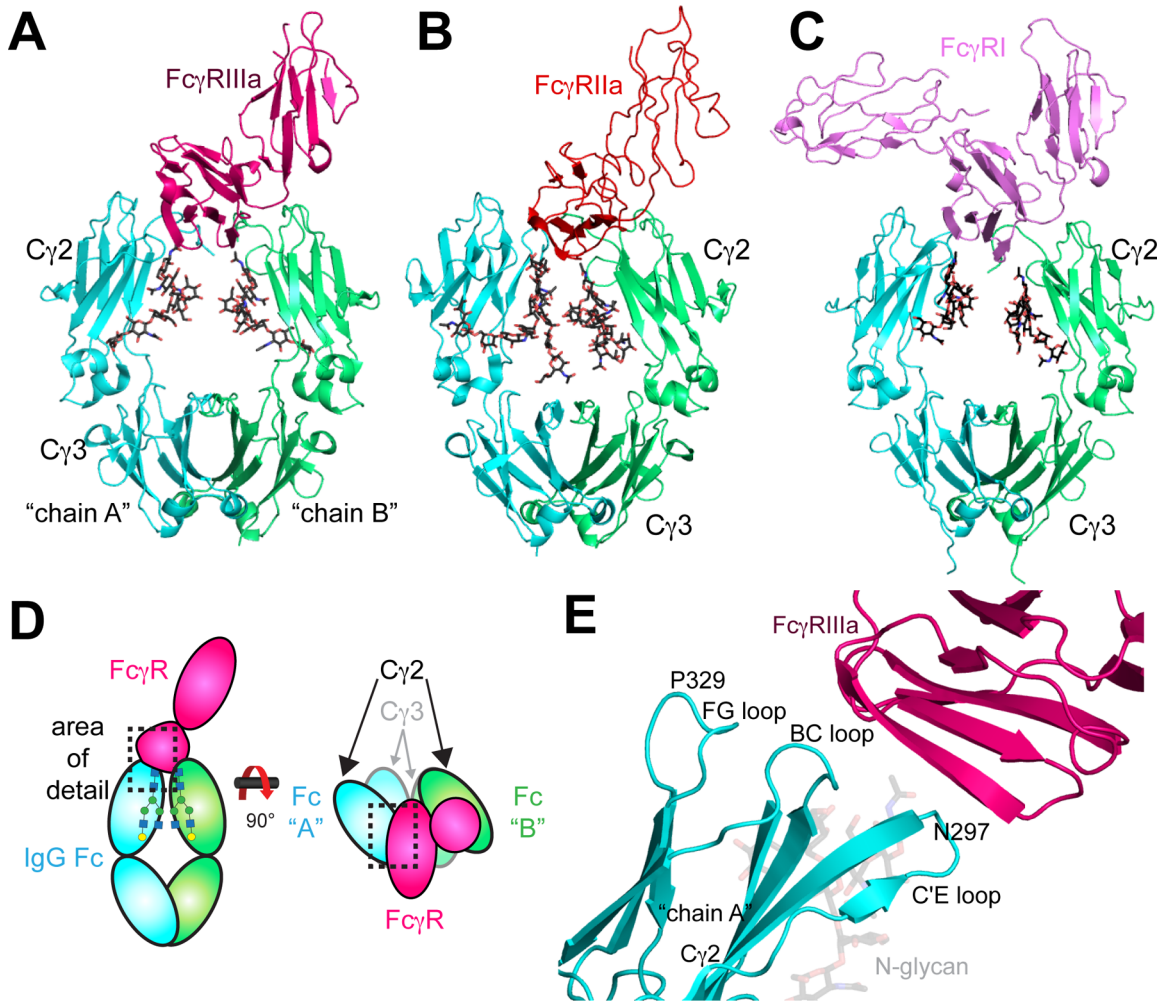

Figure 2. Structural models of (A) the Fc−FcγRIIIa interaction (PDB entry 3AY4), (B) the Fc−FcγRIIa interaction (PDB entry 3RY6), and (C) the Fc−FcγRI interaction (PDB entry 4X4M). Fc N-glycans are shown as black stick models. (D) Schematic diagram of the Fc−FcγR interaction. (E) Fc Cγ2 loops serve as the FcγRIIIa (pink) binding site. The C′E loop includes N297, the site of N-glycosylation. The Fc chain A monomer (cyan) is shown to emphasize the locations of the loop structures (PDB entry 1E4K). The interaction between chain A and FcγRIIIa occurs primarily at the site of these Fc Cγ2 loops.

IIb. Cγ2−Cγ3 Interface. Two features likely contribute to restricting Cγ2 orientation. The disulfide-bonded hinge links the Cγ2 domains at the end of the Cγ2 distal to the pivot point formed by the Cγ2−Cγ3 domain interface. This interface likewise restricts the overall domain motions of the Cγ2 domains.[8,20] The Cγ2−Cγ3 interface is stabilized through two salt bridges, hydrogen bonds, and a hydrophobic "ball-in-socket" joint (Figure 3). E380−K248 and E430−K338 salt bridges are poised to restrict Cγ2 orientations. This hypothesis is supported by 200 ns MD simulations of the Fc E380A/E430A mutant that revealed increased flexibility of the Cγ2 domains compared to the flexibility of those of wild-type Fc.[8] Interestingly, The E380A mutant by itself appears to affect only FcRn binding, while the E430A mutant reduces affinity for only FcγRIIIa.[32] The side chain atoms of L251 form the ball of the "ball-in-socket" joint and are found at the Cγ2−Cγ3 interface (Figure 3).[20]

IIc. N297 Glycan. The structural role of conserved IgG1 Fc glycosylation at N297 is a topic of great interest. Therapeutic mAbs largely require appropriate N-glycosylation for activity, complicating drug manufacture as recombinant bacterial expression hosts do not express proteins with mammalian N-glycans. Furthermore, the Fc N-glycan is heterogeneous with respect to composition.[33−35] The variability in Fc glycan composition, and glycans in general, originates because complex carbohydrate biosynthesis is not a template-driven process, unlike protein and nucleic acid biosynthesis. Compositional glycan diversity results from conserved, but variably complete, modifications by glycosyltransferases and glycosylhydrolases in the endoplasmic reticulum and Golgi complex.[36,37]

Two aspects of the Fc N-glycan composition indicate that it contributes to Fc interactions. The distribution of Fc N-glycan compositions changes in certain disease states, and changes in Fc N-glycan composition affect affinity for many FcγRs (both topics are covered in detail below). The Fc N-glycan is primarily of a biantennary, complex type with a high level of core fucosylation [>95% (see Figure 4)]. A common motif found in all human Fc N-glycan structures is a heptasaccharide composed of chitobiose linked to N297 followed by a branching trimannose structure with terminating N-acetylglucosamine (GlcNAc) residues on the nonreducing branch termini (Figure 4). The N-glycan can be further decorated with terminal galactoses and then sialic acids, and occasionally the addition of a bisecting GlcNAc residue. In general, the IgG

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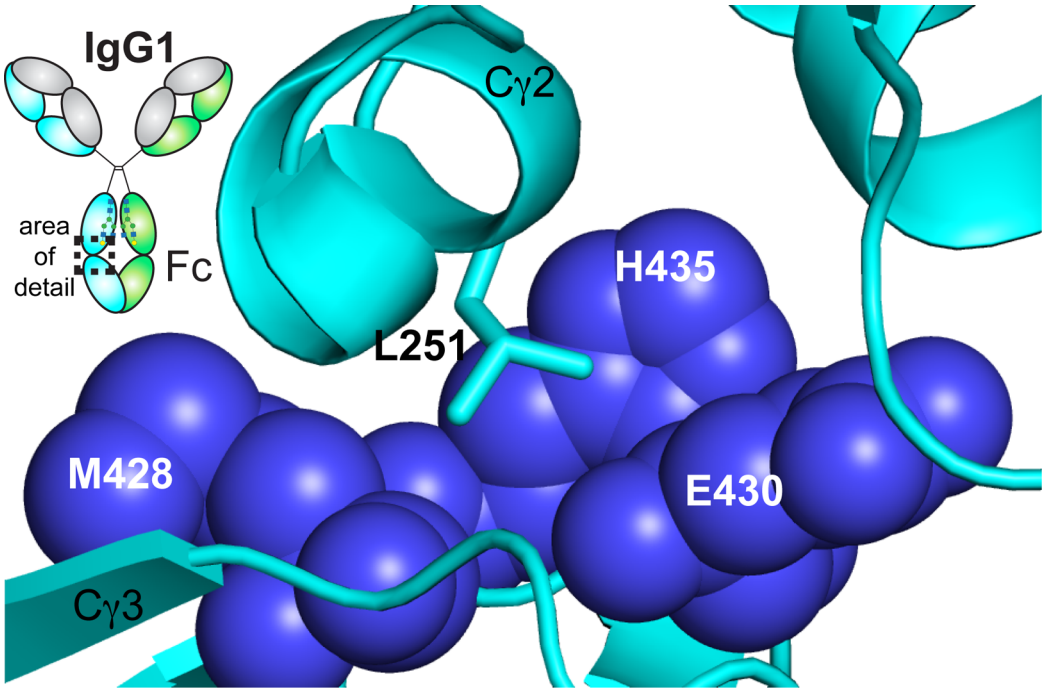

Figure 3. L251 (cyan stick model) forms the pivot point in a "ball-in-socket" joint that guides Cγ2 motions. E430, H435, and M428 of the Cγ3 domain form the socket (blue spheres).

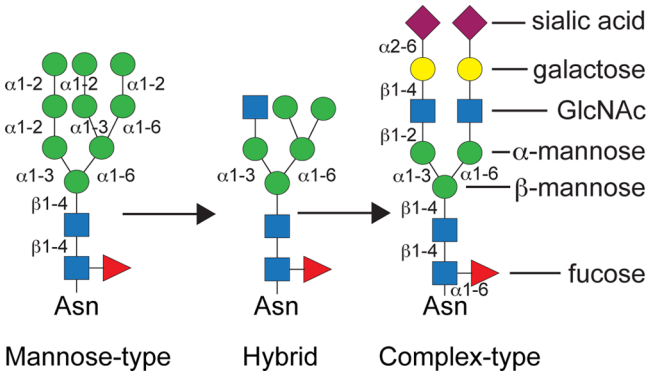

Figure 4. Fc N-glycan maturation. The most common forms of the Fc N-glycan include zero, one, or two galactose residues and zero or one sialic acid residues.

Fc N-glycan is subject to less processing than most N-glycans, because of the interaction between N-glycan and polypeptide residues. In serum from healthy human subjects, the Fc N-glycan ranges from 30 to 35% GlcNAc-terminated, ~50% Gal-terminated, and 10−15% sialic acid-terminated.[38] The branched structure of the N-glycan is characterized by the linkage between the β-linked mannose residue at the branch point and the α-linked mannose residues that form the first residues of the branches at the nonreducing termini of the N-glycan. One of these branch mannose residues is linked by an α1−6 linkage, and this forms the "6-arm" of the N-glycan. The other is linked through an α1−3 linkage and forms the "3-arm" of the N-glycan.

Glycan changes have been noted in multiple diseases, but rheumatoid arthritis (RA) is of particular note. In advanced RA patients, the GlcNAc-terminated (G0F) form dominates.[39,40] Changes in the glycan distribution predate RA onset by as many as 3.5 years,[41] and changes in the Fc N-glycan composition of RA patients temporarily return to normal during pregnancy-induced remission.[42] One hypothesis for explaining the correlation between RA and glycan composition is that the G0F glycoform is more pro-inflammatory than the galactosylated form.[43] If galactose termini were available, a small percentage of Fc would be transformed to a sialylated form, which is believed to be potently anti-inflammatory (discussed further below). In either case, it is clear that Fc glycosylation at N297 is vital to the proper function of IgG1 Fc.

Aglycosylated or deglycosylated Fc does not bind the low-affinity Fc γ receptors (IIa, IIb, IIIa, and IIIb),[44−46] but binding of the high-affinity receptor, FcγRI, is preserved.[14,47] Glycan composition likewise impacts the affinity of Fc for FcγRs.[24,31,38,48−52] One well-described example is fucosylation of the (2)GlcNAcβ1−4(1)GlcNAcβ-N297 core chitobiose disaccharide that decreases the affinity of Fc for FcγRIIIa by 10−50-fold.[53−56] Fc structures determined by X-ray crystallography revealed that Y296 becomes solvent-exposed in fucosylated Fc,[57] disrupting a contact between an FcγRIIIa N-glycan and the Fc N-glycan.[16,58]

Changes to the nonreducing termini of the glycan (distal to N297) also impact receptor binding.[59] For example, the presence of GlcNAc and galactose residues at the Fc N-glycan termini improves the affinity of Fc for FcγRIIIa.[31] Addition of these residues enhances interactions between the Fc N-glycan and polypeptide surface, likely stabilizing an Fc conformation that is predisposed to FcγR binding.[60,61]

An unexpected feature of N-glycan composition and its effect on FcγR affinity was revealed recently by Subedi et al. Though the N-glycan is required and changes to the termini affect affinity, it was noted that Fc, trimmed back to a glycan that consists of only a single GlcNAc residue, still binds FcγRIIIa with an ~10-fold reduction in affinity when compared to that of Fc with a full-length G2F N-glycan.[62] This result suggests that the (1)GlcNAc residue alone provides the predominant contribution to N-glycan−FcγRIIIa binding, consistent with similar measurements of N-glycan contributions to intra-

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molecular stability.[63] The observation that (1)GlcNAc-Fc binds was surprising because aglycosylated Fc, as noted above, does not bind FcγRIIIa, nor is Fc enzymatically cleaved to contain an N-glycan of the (1)GlcNAc and (0)fucose residues.[31,64] The latter observation can be understood by considering the additional 10−50-fold negative impact of fucosylation on FcγR binding, as noted above, likely pushing the association of the Fc with a fucose-GlcNAc disaccharide beyond detection limits.

The effect of sialylation on Fc structure and Fc-mediated interactions is an open question and of great interest because sialyl-Fc was reported to be a keen mediator of an anti-inflammatory response with therapeutic potential.[65] Sialylation of the Fc N-glycan, a less abundant modification in healthy human serum at ∼5−10%, was reported to reduce the affinity of Fc for FcγRIIIa by 10-fold when the reaction was enzymatically pushed to completion and upon formation of high titers of disiaylyl Fc.[33,65,66] However, these results have been challenged by other observations that found no change in binding affinity caused by sialylating wild-type Fc.[14] It was proposed that sialylation shifted Fc specificity to favor an anti-inflammatory receptor, DC-SIGN;[67−69] however, these results have also been recently challenged.[70] Structures of sialyl Fc were also reported and were found in two different forms: one much like other Fc structures showing no large-scale structural consequence of sialylation[13] and another showing some rearrangement of the Cγ2 domain orientation.[21] This area of inquiry is nascent and without a clear definition of the effects of Fc sialylation, but it is clear that the behavior of the Fc N-glycan is complex.[29]

III. GLYCAN MOTIONS: THE FC N-GLYCAN IS DYNAMIC

IIIa. Motion of the Fc N-Glycan. The Fc N-glycan was long thought to remain bound to the Fc polypeptide surface between the two Cγ2 domains of the homodimer based on X-ray crystallography structures.[6,7] This conformation would lead to steric occlusion of the N-glycan termini and restriction from N-glycan-modifying enzymes. However, the Fc N-glycan is sensitive to enzymatic modifications in the Golgi and in vitro, suggesting the Fc N-glycan must populate exposed conformations at some frequency.

A clear relationship between glycan composition and glycan function has long been of interest, with conflicting results having been published over the years. Motion of the Fc N-glycan was first thought to be on the same time scale as Cγ2 domain motion, suggesting the N-glycan was bound to the Cγ2 surface.[71−73] Wormald and colleagues later measured relaxation rates of the IgG1 Fc N-glycan by solution nuclear magnetic resonance spectroscopy (NMR) and noted that they were lower than those of the bulk protein, suggesting that the glycan was mobile.[74] Shortly after this study, Kato and colleagues used a [^{13}C]galactose labeling strategy to measure spectra of the galactose residue at the nonreducing termini of Fc and found a similar result for the galactose residue on the 3-arm of the Fc N-glycan, indicating a high degree of mobility relative to the Fc polypeptide.[28] In contrast, the ^{13}C label in the galactose residue on the 6-arm of the glycan revealed a line shape that was much broader and similar to that expected for a Cα atom, indicating restriction by the polypeptide as would be expected on the basis of the location of the 6-arm galactose residue according to structures determined by X-ray crystallography.

The findings of Yamaguchi et al. regarding immobility of the 6-arm of the N-glycan were challenged by Barb and Prestegard, who used Fc remodeled with [$^{13}C_2$]galactose to thoroughly characterize the motion of the Fc N-glycan using solution NMR spectroscopy. These studies revealed that surprisingly both branches of the Fc N-glycan were mobile and experienced significant motion at physiological temperatures.[29] Slow, microsecond motions of 6-arm galactose resonances contributed to the broad [^{13}C]galactose line shapes and thus explained the apparently conflicting results reported by Yamaguchi et al. The NMR data of Barb and Prestegard revealed that the 3-arm experienced one highly mobile state, while the 6-arm exchanges between two states on a microsecond time scale: one dominant, highly mobile, unrestricted state and a minor polypeptide-bound, restricted state.

IIIb. N-Glycan Motion Is Perturbed by Fc Mutations. Together, data collected using NMR and X-ray crystallography provide a model of N-glycan motion. Interactions between polypeptide and N-glycan residues restrict the motion of the N-glycan termini; however, the restriction of 6-arm residues is significantly greater than that of the 3-arm because of more extensive intramolecular contacts near the nonreducing terminus of the 6-arm. In addition, the intramolecular interactions between the Fc polypeptide and N-glycan restrict the degree of glycan motion.[62] Residues F241, F243, D265, V264, K246, and R301 were identified as key residues in the glycan−polypeptide interaction.[14,62,75] Mutations at these sites disrupt the interaction and increase the extent of glycan processing in the Golgi. X-ray crystallography indicates that disrupting these interactions has a small impact on Fc conformations sampled by Fc, potentially altering its ability to interact with receptors.[14,21]

Mutations of aromatic residues at the interface formed by N-glycan and polypeptide residues were designed to abrogate π-CH interactions, thought to be the predominant force behind many strong carbohydrate binding sites.[76] The F241A mutation is designed to disrupt the interaction between F241 and (2)GlcNAc.[75] Experimental structures of the F241A mutant have been determined by X-ray crystallography.[14,21] Though the structures are largely similar to those previously observed, it was noted that electron density of the 3-arm is reduced in the F241A mutant,[14] suggesting an increased level of N-glycan motion. F241A, F241I, F241S, F243I, and F243S Fc mutants likewise show greater levels of sialic incorporation, which is likewise consistent with an increased level of motion and increased accessibility.[14,62]

IIIc. Association of Fc N-Glycan Motion and FcγRIIIa Affinity. A quantitative analysis of N-glycan motions using NMR determined that the 6-arm of Fc F241S was significantly more mobile than that of wild-type Fc.[62] Increases in the level of glycan motion were likewise observed with F241I, F243S, F241I/F243I, and F241S/F243S mutants.

Glycan motion was found to be correlated with FcγRIIIa affinity.[62] Residues F241, F243, and K246 were mutated to perturb the Fc glycan−polypeptide interaction. Fc F241I/F243I and Fc F241S/F243S double mutants had considerable decreases in the level of glycan restriction with 20- and 60-fold decreases in FcγRIIIa affinity, respectively. Fc F241I, Fc F241S, and Fc F243S showed less perturbation of glycan restriction, and FcγRIIIa binding was intermediate between those of wild-type Fc and Fc double mutants (4-, 3-, and 4-fold reduced affinity, respectively). Fc K246F appeared to stabilize the Fc N-glycan, reducing mobility, while promoting FcγRIIIa

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interaction. A comparison of the glycan motion versus FcγRIIIa affinity revealed a strong linear correlation between the two parameters.[62]

IIId. Motion of Sialylated N-Glycoforms. As noted above, sialyl Fc is potentially potently anti-inflammatory;[38,43,65,77−79] thus, it was of interest to determine if sialylation modified the structure of the Fc N-glycan. Measurements by solution NMR spectroscopy found little change in the motion of the N-glycan upon sialylation of the 3-arm, or both 3- and 6-arms.[80] This is consistent with more recently determined structures of sialyl Fc showing no contact between the 6-arm sialic acid and the Fc polypeptide.[13,21]

Sensitivity of Fc to modification by ST6Gal-I, the primary α2−6 sialyl transferase in humans, provides information about global motions of the N-glycan. The sialyl transferase St6Gal-I adds sialic acids to galactose-terminated N-glycans.[81−84] ST6Gal-I has a branch preference for the 3-arm of the Fc N-glycan, even when the glycan is released from the polypeptide surface.[81,85,86] The conservation of relative branch modification by ST6GalI was similar for Fc-conjugated and free N-glycans, indicating the innate branch specificity of ST6GalI was not influenced by the Fc polypeptide.[80] This result suggested the Fc N-glycan samples conformations that have both branch termini either simultaneously exposed or restricted from access by the enzyme.

IV. FC−FC RECEPTOR INTERACTIONS

Interaction between the Fc region of immunoglobulins and Fc receptors links the humoral and cellular immune responses. The IgG Fc receptor family is comprised of one high-affinity receptor (nanomolar affinity), FcγRI, and several low-affinity receptors (micromolar affinity), FcγRIIa, FcγRIIb, and FcγRIIIa.[1−4] FcγRs are, in general, activating receptors except for the inhibitory FcγRIIb. Fc can also trigger responses through interactions with TRIM21 and C1q, and Fc has been associated with DC-SIGN. Maintaining antibodies in the serum, preventing degradation, and transcytosis of IgG across the placenta are mediated by an additional interaction of Fc with the neonatal Fc receptor, FcRn.[87,88] The ability to target specific receptors is desirable for imparting response specificity in future antibody-based biotherapeutics. In this section, we discuss the current evidence and models of these interactions.

IVa. FcγRIIIa (CD16). High-resolution models show how one FcγRIIIa protein binds to one Fc dimer, in an asymmetric interaction that occupies the lower hinge region, the BC loop, the C′E loop (containing N297), and the FG loop of Fc (Figure 2).[16,27,89−91] The extracellular domains of FcγRIIIa and FcγRIIIb are 97% identical, and IIIa has a 21-residue C-terminal extension. Thus, the binding of IIIa and IIIb is expected to be identical. The contact surface area between Fc and FcγRIIIa varies between 1200 and 1700 Å^2,[16,91] including both protein−protein and protein−carbohydrate interactions. S239 and L235 on both Fc chains form contacts with FcγRIIIa. Fc residues 327−330 on only one chain contact FcγRIIIa, as shown by high-resolution structures and functional analysis of mutant proteins.[9,10,16,92]

While it is easy to disrupt receptor binding though mutation, several studies demonstrated an increased level of binding, including that of the Fc S239D/A330L/I332E variant that increased FcγRIIIa affinity 30-fold.[10] Another Fc variant, L234F/L235E/P331S, has impaired affinity for FcγRIIIa and other FcγRs.[9] Most likely, this reduction in affinity is due to the L235E mutation, which replaces a hydrophobic contact with a highly charged group.

Recent approaches engineering Fc for maximal FcγR affinity include breaking Fc symmetry to capitalize on the asymmetry of the Fc−FcγR complex (Figure 2D). The majority of the FcγRIIIa interaction occurs between only one Fc heavy chain monomer (hereafter termed the "A" chain).[16,27,91] The asymmetric binding mode indicates that creating a synthetic heterodimer of different heavy chain polypeptides could more specifically influence the interaction between Fc and FcγRIIIa.[93−96] For example, introducing a set of four alterations into the A chain of Fc (D270E, K326D, A330K, and K334E) and seven into the B chain (L234Y, L235Y, G236W, S239M, H268D, S298A, and A327D) improves binding to FcγRIIIa 1000-fold.[95,96] Interestingly, the structure of the A chain is not dramatically perturbed, having a backbone root-mean-square deviation (rmsd) of 0.67 Å in the Cγ2 domain versus structures of only Fc.[96] The B chain, on the other hand, is more affected with an rmsd of 1.13 Å for the Cγ2 domain versus wild-type Fc.

In addition to the role of Fc N-glycosylation in Fc−FcγR interactions (see section IIc), FcγRIIIa N-glycosylation is also a measurable, but not required, factor. There are five glycosylation sites on FcγRIIIa. Of these sites, only N162 and N45 appear to be important for Fc−FcγRIIIa interactions. As mentioned previously, fucosylation of Fc blocks the FcγRIIIa N162 glycan from interacting with Y296 of Fc.[16,57] This interaction is specific for the N162-linked glycan on FcγRIIIa as removal of the glycan promotes interactions with fucosylated Fc.[97] The N45 glycan is thought to have an inhibitory effect on binding; removal of the N45 glycan promotes Fc−FcγRIIIa interaction.[97,98] One theory for the inhibitory effect of the N45 glycan is that steric interference between the N45 glycan and chain B of Fc blocks Fc−FcγRIIIa interactions.

IVb. FcγRII (CD32). FcγRIIa functions as an activating receptor, and FcγRIIb inhibits immune responses.[1,2,99] This functional difference is due to the presence of a cytosolic immune receptor tyrosine activating motif (ITAM) in FcγRIIa and an immune receptor tyrosine inhibitory motif (ITIM) in FcγRIIb. While FcγRIIa and FcγRIIb are functionally distinct, their extracellular domains are structurally similar.[100−102] At the amino acid level, the sequences of the extracellular domains of FcγRIIa and FcγRIIb are 89% identical. This high degree of similarity is maintained in the folded proteins. Alignment of FcγRIIa and FcγRIIb structural models reveals an rmsd of only 1.1 Å (Figure 5). Despite the high degree of similarity, Fc variants that show isotype specificity are described.[32,94]

The mechanism of engagement of FcγRII by Fc was initially unclear. Isolated FcγRIIa and FcγRIIb both crystallize as dimers.[100,102] This observation led to the speculation of a 1:2 Fc−FcγRIIa complex that was reportedly supported by computational modeling of the Fc−FcγRIIa interaction.[101] However, costructures of Fc with FcγRIIa showed a single FcγRIIa receptor bound at the lower hinge region of IgG Fc, much like the previously mentioned FcγRIIIa.[17] Sedimentation equilibrium centrifugation, isothermal titration calorimetry, and NMR experiments confirmed the binding stoichiometry was 1:1.[103−106] However, it should be noted that FcγRIIa is thought to exist as a dimer on the surface of cells, and the *in vivo* characteristics of the complex have not been thoroughly characterized.[17]

IVc. FcγRI (CD64). The structure of the extracellular domains of FcγRIa has recently been determined.[107] There are several differences between FcγRI and the rest of the FcγR

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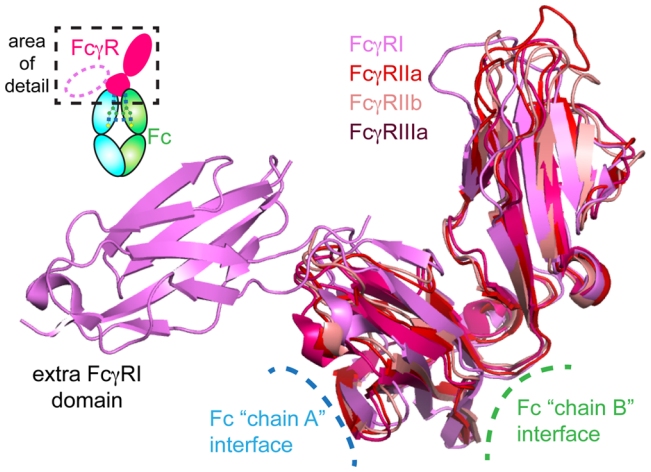

Figure 5. High level of structural conservation among FcγRs. Ribbon diagrams highlight the interfaces with IgG Fc and the extra FcγRI domain. PDB entries 3RJD for FcγRI, 1FCG for FcγRIIa, 2FCB for FcγRIIb, and 3AY4 for FcγRIIIa.

family, including affinity (I ≫ II ∼ III), and FcγRI binds aglycosylated Fc with high affinity (high nanomolar). Furthermore, FcγRI can bind monomeric Fc on cell surfaces, unlike FcγRII and FcγRIII that signal only following Ig-dependent clustering of FcγR molecules on the cell surface.[108]

Higher affinity is not the only feature that distinguishes FcγRI. FcγRI has a prominent third extracellular domain, which is not present in FcγRII or FcγRIII.[109] Early research suggested the third domain was responsible for improved affinity in mice.[110] In this study, removal of the third domain in FcγRI removed the high-affinity recognition of Fc. An experiment in mice revealed that including the third domain in a low-affinity receptor transformed the low-affinity receptor into a high-affinity receptor. Later, the second domain was also identified as playing a role in promoting high-affinity interactions between FcγRI and Fc.[111] Recent studies support the role of the second domain in increasing the level of binding and contest the importance of the third domain.[107,112] Residues 171−176 of FcγRI form the FG loop in FcγRI. The FG loop is located in the second extracellular domain of FcγRI and forms a perfect binding wedge to bridge both Fc Cγ2 domains.[113] Swapping the FcγRI FG loop for the same residues in FcγRIIIa increases the affinity of FcγRIIIa for Fc 15-fold.[107]

The importance of the FG loops is highlighted in the recent report of a high-resolution structure of the FcγRI−Fc complex.[113] Two key features make the FcγRI FG loop suitable for Fc interactions: the shorter length of the FcγRI FG loop and the presence of a positively charged KHR motif. The FG loop in FcγRI is one residue shorter than the corresponding loop in FcγRII or FcγRIII. In FcγRIII, the FG loop was found to bend away from the Fc glycans to accommodate the additional residue. However, in FcγRI, the FcγRI FG loop is 5 Å closer to Fc. This may permit a tighter interaction between the FcγRI FG loop and Fc. This position alone likely does not completely explain the greater FcγRI affinity. The KHR motif in the FcγRI FG loop is positively charged. This allows for the formation of additional contacts between FcγRI and Fc. The positive charges on the FcγRI FG loop are important for forming salt bridges between K173 of FcγRI and D265 of Fc. Swapping any of the residues in the KHR motif for neutral or negatively charged amino acids results in 2−30-fold decreases in affinity.[113]

IVd. DC-SIGN. DC-SIGN is an inhibitory receptor on dendritic cells and macrophages that interacts with high-mannose-type glycans to recognize pathogens. While this is not a traditional Fc receptor, it was proposed that interactions with DC-SIGN explain the anti-inflammatory effects of sialyl-Fc.[65,69] Removal of SIGN-R1, a DC-SIGN homologue in mice, abrogates the restorative effects of intravenous treatment with donated immunoglobulins (IVIg), but adding human DC-SIGN restores its functionality.[78,114] It was suggested that Fc sialylation induces a structural change to unveil a new epitope recognized specifically by DC-SIGN.[67]

Similar to the anti-inflammatory properties and structure of sialyl Fc, the interaction between sialyl Fc and DC-SIGN remains an unresolved topic in the literature. A small number of published studies refute the formation of a complex between sialyl-Fc and DC-SIGN. The strongest evidence supporting this view is that DC-SIGN, a C-type lectin, does not bind sialylated N-glycans in carbohydrate binding arrays.[115] One study using carbohydrate arrays shows that sialylation of certain epitopes, like Lewis X, in fact prevents interaction with DC-SIGN.[116] Furthermore, sialyl Fc does not compete with DC-SIGN ligands in carbohydrate binding experiments and binds no better than deglycosylated Fc.[70] One theory is Fab cross-reactivity, and not Fc sialylation, allows IVIG to interact with DC-SIGN.[70] No structures of the Fc−DC-SIGN complex are available as of the writing of this review.

IVe. FcRn. The neonatal Fc receptor (FcRn) is structurally and functionally unique among Fc receptors. Named initially after its role in transporting IgG across the placenta, FcRn is not known to serve a signaling function. Rather, FcRn is responsible for transcytosis of IgG and recycling endocytosed IgG back to the serum.[1,88,91] FcRn is similar in structure to the major histocompatibility complex molecules.[117] FcRn interacts with the Cγ2−Cγ3 interface of Fc, and not the lower hinge region like FcγRs[118] (Figure 6A). The interaction between Fc and FcRn is pH-dependent, exhibiting tight binding to Fc at pH 6 and weak binding at pH 7.4 This pH dependence allows for tight binding between FcRn and IgG in lysosomes and then release of IgG in neutral environments. In mice, the pH dependence is thought to be due to Fc H435−FcRn E132, Fc H436−FcRn D137, and Fc H310−FcRn E117 salt bridges (Figure 6B). In humans, the salt bridge pairs between H435 and E132 and between H310 and E117 still exist, but residue 436 is a tyrosine.

In principle, the serum half-life of Fc-based therapeutics can be improved by engineering Fc to bind more tightly to FcRN at pH 6.[119] This has been achieved through an Fc YTE variant (M252Y/S254T/T256E) that binds FcRn with a 10-fold greater affinity, but at the cost of a 2-fold decrease in affinity for FcγRIIIa.[119,120] The structure of this Fc is largely conserved, with an rmsd of only 0.86 Å when compared to wild-type Fc;[11] however, S254 mutations are known to impair FcγRIIIa interactions.[32]

Additional Fc variants are reported to enhance the Fc−FcRn interaction.[32] Fc T307A/E380A/N434A demonstrates a 16-fold increase in the level of binding to isolated FcRn and a 3.3-fold increase in the level of binding to cells expressing FcRn.[121] T250Q/M428L has a 30-fold increase in serum half-life that is pH-dependent.[122] The Fc H433K/N434F variant also has a 16-fold increase in affinity for FcRn at pH 6.0 but unexpectedly revealed a 4-fold reduction in half-life in mice.[123] Fc variants that increase FcRn affinity without affecting other FcR interactions likewise would be useful for increasing effi-

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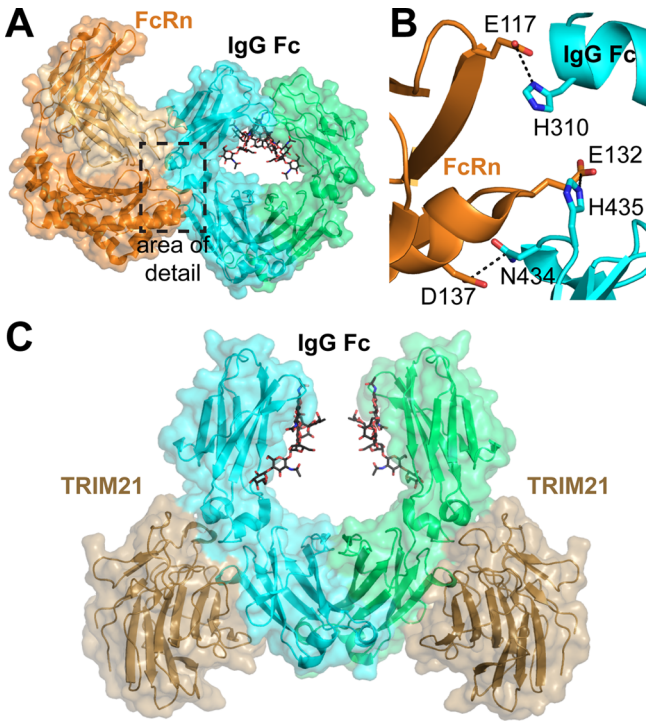

Figure 6. (A) Neonatal Fc receptor (FcRn, orange ribbon) that binds to the Cγ2−Cγ3 interface of IgG1 Fc (cyan ribbon) (PDB entry 1I1A). (B) Key H-bond and ionic interactions formed between Fc and FcRn that are responsible for the pH dependence of binding. Fc residues 245−260 were removed from this image for the sake of clarity. (C) PRYSPRY domain of TRIM21 (sand) that recognizes the Cγ2−Cγ3 interface of IgG Fc (cyan, *green*) in a manner similar to that of FcRn (PDB entry 2IWG).

cacy.[88,124] Those that do have impaired affinity for other FcRs are useful as Abdegs, antibodies that promote the degradation of pathogenic Igs by preventing their recycling by FcRn.[125]

IVf. TRIM21. TRIM21 is a member of the tripartite motif family of pathogen defense proteins and binds Fc. TRIM21 contributes an important function in viral defense by binding to intracellular IgG−virus complexes. TRIM21 marks these complexes for degradation by the proteasome, destroying the virus and bound antibody.[126] It should be noted that enveloped viruses can shed IgG before infecting a cell and thus do not initiate a TRIM21-mediated response. Additionally, the antiviral capabilities of TRIM21 can be overcome by super-infection.[127] However, knockout studies in mice have shown that TRIM21 is necessary for antibody-dependent intracellular neutralization.

TRIM21 has a low nanomolar affinity for IgG and is able to compete with protein A for Fc binding.[127−130] TRIM21 binds to Fc at the Cγ2−Cγ3 interface in a manner similar to that of FcRn (Figure 6).[126] This binding location allows two TRIM21 proteins to interact with one Fc.[129,130] The TRIM21−Fc interaction is mediated by ionic interactions. Despite the similarity between the Fc motifs recognized by TRIM21 and FcRn, the TRIM21−Fc interaction does not appear to be pH-dependent.[130]

IVg. General Theories of the Fc N-Glycan Requirement. Of the receptors discussed above, the low-affinity FcγRs and (potentially) DC-SIGN require Fc N-glycosylation, while TRIM21 and FcRn do not. Two hypotheses have been put forth to describe the structural consequence of Fc N-glycosylation in low-affinity FcγR binding.[62] This is still very much an open question. One prevalent theory, based primarily on models determined by X-ray crystallography, suggests glycan composition affects the relative orientation of the Fc Cγ2 domains.[15,25,67,68] According to this hypothesis, the Cγ2 domains of Fc sample a range of conformations, some predisposed to bind FcγRs with the receptor binding site easily accessible and others populating conformations that are unfavorable for Fc−FcγR interactions. In this model, glycan composition tunes the Cγ2 orientation, with pro-inflammatory glycoforms assuming a small set of conformations predisposed to bind receptor and truncated or aglycosylated forms incapable of binding. Computational simulations of Fc motions are consistent with this hypothesis.[8] One primary limitation of models built from X-ray crystallography data is that only the low-energy forms are observed, and the low-energy forms may not be highly populated in solution at physiological temperatures. Indeed, X-ray crystallography is blind to the predominant highly mobile state of the Fc N-glycan termini as discussed above (section III)

An alternative hypothesis built upon solution measurements suggests local structural perturbations explain differential receptor binding affinities. This idea was first suggested by Jefferis and co-workers with data directly supporting this idea by the groups of Kato and Barb and developed further by Barb.[57,62,75] In this model, the role of the Fc N-glycan is to restrict local Fc conformation, including the C′E loop. This is an attractive hypothesis because N297, the site of N-glycan attachment, sits at the apex of the C′E loop; furthermore, significant contacts are made between FcγRII and FcγRIII and the Fc C′E loop.

The two models describing the role of the Fc N-glycan are not mutually exclusive. Solution NMR studies using molecules of this size (∼55 kDa) are incapable of providing high-resolution definitions of all atoms in the system, unlike X-ray crystallography, and may be blind to certain features and certain time scales of motion. Defining which of these models best accounts for the predominant forces behind the contribution of N-glycan to FcγR binding will be informative for future targeted improvement of immunoglobulin G-based therapeutics.

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Notes

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