CHAPTER

10

Antibody Glycosylation

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The antibody Fc region is posttranslationally modified by *N*-linked glycosylation. In immunoglobulin G (IgG), the processing of the glycans is restricted by the presence of extensive interaction with the protein surface. The resulting set of antibody glycoforms exhibit a range of effector functions. In this chapter, we outline the impact of glycosylation on the immune function of antibodies and discuss the implications for monoclonal antibody and intravenous immunoglobulin therapies.

HUMAN ANTIBODY: AN OVERVIEW OF STRUCTURE AND FUNCTION

Immunoglobulins (Igs), also called antibodies, are an important component of the human immune system. The multiple isotypes of antibodies differ between different animal species, varying from seven in horses to only one in rabbits. Human antibodies are classified into five categories based on their distinct structure, function, and location: IgA, IgD, IgE, IgG, and IgM, of which IgG is the most investigated and thus most well characterized to date.[1,2] IgG is a relatively large glycoprotein with an overall Y-shaped structure comprised of two heavy chains, each approximately 50 kDa, and two light chains, each approximately 25 kDa. Together, the heavy and light chains form three distinctive regions: the antigen binding region (Fab), the crystallizable region (Fc), and the hinge region. Fab binds a specific antigen, and Fc bridges the innate and adaptive immunity by binding to Fc receptors (FcRs) as well as components of the complement system. The hinge region serves to link the Fab and Fc regions. There are two well-established functions for antibodies in the immune system. First, they bind to antigens, such as foreign pathogens, to form an immune complex to neutralize their pathogenicity. Second, the Fc region of the immune complex can bind to FcRs or complement components to clear pathogens and trigger both innate and adaptive immune responses.

Different antibody isotypes, which mediate distinct functions, are produced at different stages of an immune response under different stimulatory milieus, via a regulated isotype

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switching mechanism in B cells.[3] By default, B cells produce IgM without previous exposure to an antigen. IgM circulates in the human serum at about 1 to 2.5 mg/mL, and its typical pentameric form is a potent activator of the complement pathway.[4] IgG represents the most abundant isotype in human serum, circulating at around 12 mg/mL, and is the main isotype produced in response to active vaccination.[4] IgE is known to bind large pathogens such as parasites and is responsible for causing hypersensitivity, including the commonly known allergic reactions.[4,5] IgA is present in human serum at about 2.5 mg/mL in its monomeric form and is predominately dimeric on the mucosal surfaces, which prevents antigens such as bacteria from breaching the mucosal system.[6,7] IgD is an ancient antibody preserved in most jawed vertebrates; however, its precise function remains unclear due to a lack of investigation.[8]

HUMAN IgG Fc STRUCTURE AND FUNCTION

Human IgG Fc has a well-defined horseshoe structure and mediates a range of effector functions, including antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, complement-dependent cytotoxicity (CDC), and IgG recycling, as well as recently described antiinflammatory activity.[4,9,10,108] The Cγ2 and Cγ3 domains from each heavy chain form two interchain disulfide bonds between the two Cγ2 domains. An *N*-linked glycan is attached to the Asn297 at the Cγ2 domain, which has been shown to influence the openness of the horseshoe structure (Figure 10.1) and Fc affinity for the FcγRs and C1q.

Human IgGs are further divided into four subclasses called IgG_1, IgG_2, IgG_3, and IgG_4, according to their relative abundance in serum. Each IgG subclass is elicited by different antigens. For example, IgG_2 is mainly induced by carbohydrate antigens. Moreover, IgG_4 is typically induced by prolonged immunization with protein antigens. The hinge region of each subclass has notable differences in flexibility due to length and amino acid composition. IgG_1 and IgG_3 have approximately the same level of flexibility despite the fact that IgG_3 has many more residues at the hinge, include 21 prolines, restricting its flexibility. The IgG_2 hinge lacks a glycine residue, which reduces its relative

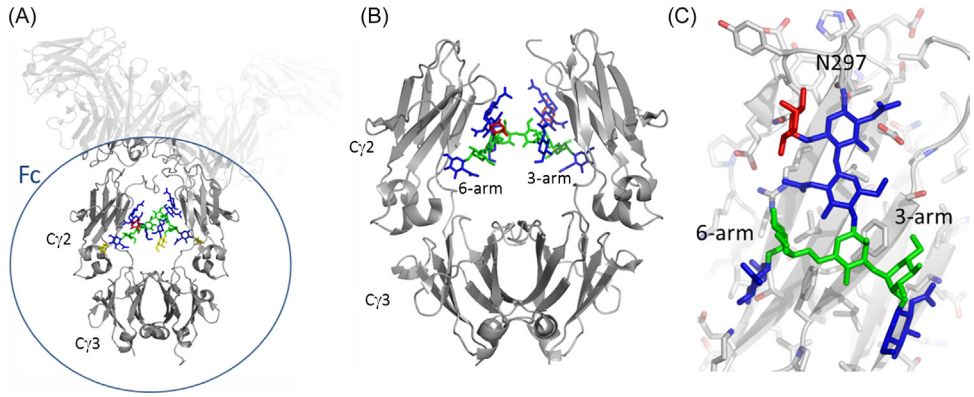

FIGURE 10.1 Crystal structure of IgG. (A) Crystal structure of an intact IgG_1 monoclonal antibody, b12 (PDB 1HZH).[85] The highlighted region corresponds to the Fc domain. (B) Crystal structure of IgG_1 Fc (PDB 3AVE).[63] (C) Structure of an *N*-linked glycan at Asn297 packing against the protein moiety of the Cγ2 domain (PDB 3AVE; chain A). Protein is displayed in gray, and glycans are displayed as colored sticks (red, fucose; blue, *N*-acetylglucosamine; green, mannose).

flexibility. IgG_4 has a key residue difference upstream of the second cysteine that normally forms the interchain disulfide bond, which is thought to account for the lack of interchain disulfide bonds for IgG_4 and provides a basis for bispecific IgG_4 generation.[11] In summary, the degree of flexibility of each subclass conferred by the hinge region is as follows: $IgG_1 = IgG_3 > IgG_2 > IgG_4$.

IgG Fc mediates antibody effector functions by binding to diverse Fc receptors on cell surfaces. These well-established receptors include FcγRs, the complement molecule C1q, and FcRn. Recently, a specific C-type lectin, DC-SIGN, has been proposed to bind to a natural population of IgG Fc capable of mediating antiinflammatory signaling,[12,13] although a direct interaction has not been supported in a biophysical study using recombinant tertrameric DC-SIGN and glycan-engineered antibodies.[14] FcγRs are type 1 single-pass transmembrane proteins; so far six human FcγRs have been cloned: FcγRIa, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa, and FcγRIIIb.[15] Moreover, they are classified as either activatory or inhibitory, depending on whether their intracellular signaling components contain the immunoreceptor tyrosine-based activation motif (ITAM) (FcγRIa, FcγRIIa, FcγRIIc, and FcγRIIIa) or the immunoreceptor tyrosine-based inhibition motif (ITIM) (FcγRIIb). FcγRIIIb is a glycosylphosphatidylinositol (GPI)-anchored receptor expressed on neutrophils. Various immune cells express multiple classes of FcγRs; for example, myeloid-derived dendritic cells and macrophages are found to express all classes of FcγRs except FcγRIIIb, in contrast to B cells and natural killer (NK) cells, which express only FcγRIIb or FcγRIIIa, respectively. The extracellular regions of FcγRs have conserved structures. Available crystal structures of FcγRs demonstrate that they have two Ig-like extracellular domains named the D1 and D2 regions.[16] Each domain is comprised of five-stranded and three-stranded β-sheets. The crystal structure of the Fc–FcγRIIIa complex shows that the interdomain angle of FcγRIIIa opens by about 10 degrees upon Fc binding. Concomitantly, Fc adopts an asymmetric opening of the horseshoe structure between the Cγ2 domains at the N-terminal tip. The Cγ2 domains and the lower hinge region of Fc form the binding site for FcγRs. Based on the structure, a high-resolution scanning of the IgG_1 Fc region has been carried out to determine the residues essential for Fc–FcγR interaction.[17] As expected due to their homologous extracellular regions, a conserved set of Fc amino acids affected binding to all FcγRs. In addition, some Fc residues were found to modulate Fc binding to only selective FcγRs, establishing a basis for selective FcγR targeting by protein engineering.

HUMAN IgG Fc GLYCOSYLATION

All human immunoglobulins are glycosylated, albeit to varying extents. IgG glycosylation accounts for 2 to 3% of total IgG molecular weight, which is relatively low compared with the 12 to 14% for IgM, IgD, and IgE. IgG Fc has a conserved *N*-linked glycosylation site at Asn297 across all subclasses. On the other hand, sequence analysis shows that only about 20% of human IgG Fab regions are glycosylated. Moreover, these *N*-linked glycosylation consensus sequences on Fab result from somatic mutations, indicating that Fab *N*-linked glycosylation is positively selected.[18,19] Indeed, most Fab *N*-linked glycosylation is shown to occur on exposed loop regions and has been reported to significantly influence antigen binding.[4] Total human serum IgG exhibits mainly biantennary, complex-type glycans, of which the neutral glycoforms represent about 86%, with agalactosylated, monogalactosylated, and digalactosylated glycoforms accounting for 25%, 35%, and 20%, respectively. Sialylated structures account for the remaining 20% (Figure 10.2). Moreover, about 30% of

IgG glycan have bisecting GlcNAc attached to the β-mannose, and between 80 and 92% of IgG glycan are core-fucosylated.[4,18,22,23] The Fc and Fab glycans differ significantly in their levels of terminal sialylation at their reducing end. As much as 80% of Fab glycan can be sialylated in contrast to about 10% of Fc glycan. A summary of human serum IgG glycosylation is presented in Figure 10.2. Interestingly, the presence of large multi-antennary IgG glycans such as triantennary glycans have not been reported in healthy people, even on the exposed Fab glycosylation sites. In contrast, other glycoproteins such as fetuin and FcγRs have been shown to contain a fair amount of triantennary and tetraantennary oligosaccharides on their solvent-exposed glycosylation sites.[24,25] This indicates that the glycosylation sites on the Fab region are not sufficiently exposed, B cells lack the enzymes required for generating multi-antennary oligosaccharide, or mechanisms may exist for specifically tuning IgG glycosylation.

The lack of terminal processing of the Fc glycan may be due to limited access of Golgi-resident glycosyltransferases as a result of limited glycan flexibility imposed by the unusually prolific glycan–protein interactions in the Fc region (Figure 10.1). The crystal structures of Fc show that the two opposing Fc glycans are positioned in the Fc horseshoe structure in

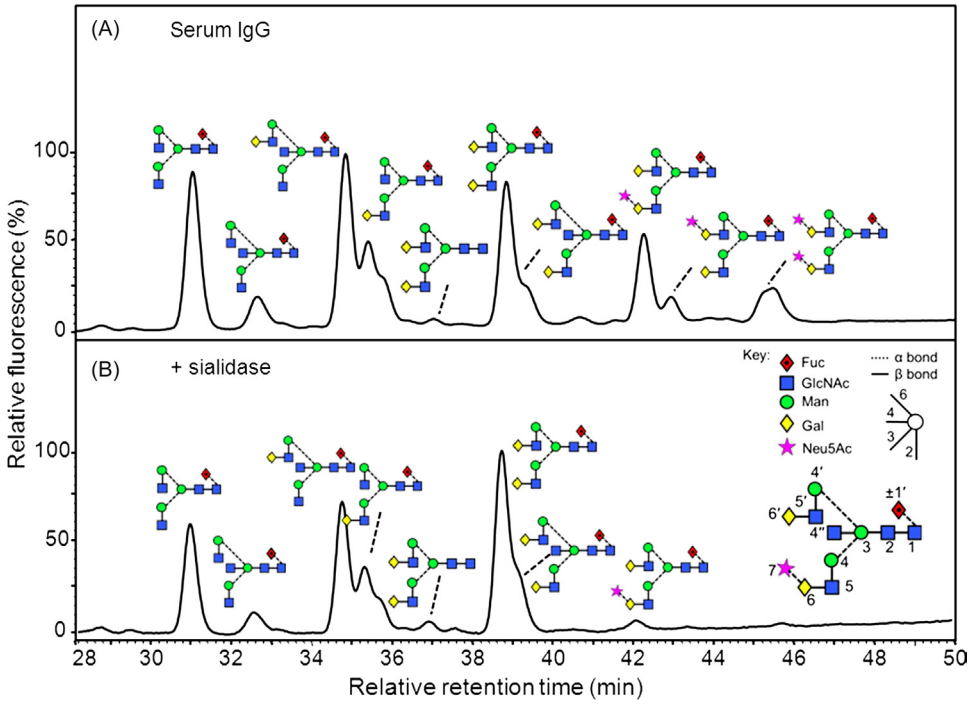

FIGURE 10.2 **Glycosylation of human serum IgG.** Normal-phase high-performance liquid chromatography of human serum IgG *N*-linked glycan fluorescently labeled with 2-aminobenzoic acid. (A) Total glycan pool. (B) Glycan profile following sialidase digestion. The symbolic representation of glycans follows that of Harvey et al.,[44] with residues in both the schematic diagrams and molecular graphics following the color scheme of the Centre for Functional Glycomics.[17] A labeled monosialylated glycan is shown to illustrate the residue labeling scheme adopted in this text. The scheme follows that of Vliegenthart et al.,[99] with the additional modifications of **7** and **7′** for sialic acid and **1′** for α1→6–linked core fucose.[31] HPLC profiles are reproduced from Yu et al.[108]

a precisely defined fashion. Each glycan lies close to a patch of amino acid residues on the Cγ2 domain, forming a number of non-covalent interactions dominated by hydrophobic and electrostatic interactions. A ladder of four hydrophobic residues, Phe241, Phe243, Val262, and V264, form CH–π interactions with the hexose rings of the core heptasaccharide and the GlcNAc on the 6-arm (Figure 10.1C). In addition, Glu265 and Arg301 form electrostatic interactions with sugar residues at the reducing end of the oligosaccharide.[30] These interactions collectively limit the Fc glycan flexibility and may result in reduced terminal galactosylation and sialylation. Moreover, this glycan immobility is also reflected in the fact that the entire oligosaccharide of the agalactosylated glycoform shows well-defined electron density in Fc domain crystal structures. Abolition of these Fc glycan–protein interactions, realized by mutating the residues along the hydrophobic ladder into alanine, significantly increased Fc terminal glycan galactosylation and sialylation.[30] In addition to the interaction between the hydrophobic ladder and the core pentasaccharide, the terminal galactose on the 6-arm has been shown to interact with the Fc protein backbone to further restrict Fc glycan flexibility. The 6-arm Fc glycan has been shown to exhibit flexibility similar to that of the Fc protein backbone,[31] while some have reported that the galactose on the 3-arm is more mobile than the Fc backbone.[32] In addition, recent NMR data show that the 6-arm has both bound and free states from the protein backbone, giving access to terminal glycosyltransferases.[33] In contrast, terminal sialylation does not influence Fc glycan flexibility.[34]

ROLE OF IgG Fc GLYCOSYLATION IN Fc STRUCTURE

The limited Fc terminal glycan processing was shown to be due to restricted access of Golgi-resident glycosyltransferases as a result of limited glycan flexibility imposed by the unusually prolific glycan–protein interaction at the Cγ2 region.[4] Early crystal structures of Fc show that the twin Fc glycans are positioned in the Fc horseshoe structure in a precisely defined fashion.[21,27,35] Each glycan lies close to a number of amino acid residues on the Cγ2 domain to which the glycan is attached, forming a number of non-covalent interactions dominated by hydrophobic and electrostatic interactions.[21,36] Moreover, the extent of twin Fc terminal glycan processing has been shown to regulate the distance between the two opposing Cγ2 domains. The successive removal of monosaccharide residues from the reducing end of Fc glycan by exoglycosidases narrows the gap between the two Cγ2 domains, while the Cγ3 domains remain unaffected.[35–37] Removal of the two terminal GlcNAc residues and the core mannoses further decreases the distance between the Cγ2 domains and lowers the Fc melting temperature, an indication of structural instability.[58,66] The impact of the Fc glycoform on Fc effector functions is discussed further below.

Crystal structures of a series of Fc glycoforms have been obtained over the years, including $Man_9GlcNAc_2$,[39] hybrid,[27] $Gal_2GlcNAc_2Man_3GlcNAc_2Fuc$,[84] $GlcNAc_2Man_3GlcNAc_2Fuc$,[21] $Man_3GlcNAc_2Fuc$,[36] $ManGlcNAc_2Fuc$,[36] and deglycosylated Fc.[36] These structures capture Fc glycoforms at different stages of glycan biogenesis, from the early oligomannose structures to the mature complex type glycans, and provide insights into the differential Fc glycan–protein packings. A comparison of these structures reveals that, regardless of the glycoform, interactions between the core pentassacharide ($Man_3GlcNAc_2$) and the Fc protein backbone are conserved.[27] One of the major changes in protein–glycan packing during Fc glycan biogenesis occurs during the transition from hybrid type to complex type. During this transition, the GlcNAc**5′** is transferred to

the 6-arm core mannose (Man**4**) by the GnTII, which allows the formation of stacking interaction between the GlcNAc**5′** and the hydrophobic residue Phe243.[27] This stacking interaction has been shown to increase Fc stability while suppressing subsequent enzymatic processing of Fc glycan.[27,41]

Apart from Phe243, which only forms significant protein–glycan interactions in complex-type Fc glycoforms, a series of hydrophobic residues comprised of Phe241, Val262, and Val264 readily forms CH–π interactions with the core pentasaccharide, regardless of the Fc glycoform.[21,30] Together, Phe241, Phe243, Val262, and Val264, in close three-dimensional proximity, form a ladder of hydrophobic residues crucial for maintaining Fc protein–glycan interactions. In addition to the hydrophobic interactions, significant electrostatic interactions occur between the positively charged Glu265 and Arg301 residues and the hydroxyl groups projecting from sugar hexoses at the reducing end of the oligosaccharide.[21,30] These hydrophobic and electrostatic interactions collectively limit Fc glycan flexibility and result in reduced terminal galactosylation and sialylation due to restricted access of Golgi-resident glycosyltransferases. Abolition of these Fc glycan–protein interactions, realized by the mutagenesis of the hydrophobic and charged residues, dramatically increased Fc terminal glycan galactosylation and sialylation, accompanied by decreased Fc-mediated cytotoxicity.[30,42–44] Several potential mechanisms have been proposed to explain this decreased Fc effector function. One possibility is that the increased Fc terminal glycan processing, particularly terminal sialylation, could alter the Fc tertiary structure to result in decreased FcγR binding. Moreover, it could be due to the putatively increased Fc glycan flexibility, as a result of reduced Fc protein–glycan interactions. In addition, a decreased glycosylation site occupancy, as observed in the hydrophobic mutants,[30] could lead to a larger fraction of aglycosylated Fc, which is known to have decreased effector functions.[17,45]

Besides the interaction between the Fc hydrophobic ladder and the core pentasaccharide of the Fc glycan, terminal galactoses have been shown to interact with the Fc protein backbone and further restrict Fc glycan flexibility. The 6-arm of Fc glycan has been shown to exhibit mobility similar to that of the Fc protein backbone,[31] an indication of the 6-arm glycan immobility. Some have reported that the Gal**6** on the 3-arm is more mobile than the Fc backbone.[32] Consistently, removal of the Gal**6′** significantly increases Fc glycan flexibility.[31] More recent NMR data show that the 6-arm of Fc glycan has both bound and free states from the protein backbone,[34] potentially permitting access to terminal glycosyltransferases. Additionally, terminal sialylation does not influence Fc glycan flexibility.[33]

ACTIVITIES OF IgG GLYCOFORMS

Deglycosylation

The Fc glycan has been shown to be essential for maintaining Fc structural stability.[27,36,37,46] As a result, deglycosylation abrogates Fc binding to all FcγRs apart from FcγRIa, which retains a tenfold decrease in binding compared to the wild type.[45,47] For example, when the Fc glycan is cleaved by the bacterial EndoS, leaving only one GlcNAc attached to Asn297, Fc binding for FcγRIIIa is abolished.[48] Moreover, mutations at the *N*-linked glycosylation site, which leads to the generation of aglycosylated Fc, abolishes Fc binding to all FcγRs.[45] On the other hand, combinations of mutations have been shown to be able to restore aglycosylated Fc binding to selective FcγRs, including FcγRIa, FcγRIIa, and FcγRIIb, albeit to a varying extent.[45] The minimal Fc glycan required for Fc

structural stability and effector function measured by FcγRI-mediated superoxide assay is found to be the core trisaccharide.[37]

Core Fucosylation

The α1,6-linked core fucose attached to Fc glycan has been established to exclusively reduce Fc binding affinity for activatory FcγRIIIa.[49,50] Defucosylation of Fc glycan increases the affinity of Fc binding affinity for FcγRIIIa by about 27-fold.[50] A comparison of the crystal structures of the fucosylated Fc–FcγRIIIa complex and defucosylated Fc–FcγRIIIa structures gives a molecular explanation for this altered affinity.[49,50] The structures showed for the first time that the *N*-glycan attached to the Asn162 of FcγRIIIa interacts directly with the core pentasaccharide of the Fc glycan. Moreover, defucosylated Fc glycan interacts more favorably with the Asn162 glycan of FcγRIIIa than its fucosylated counterpart, thus explaining the reduced affinity of fucosylated glycans (Figure 10.3). Defucosylated IgG Fc has been shown to have enhanced ADCC activities.[52–55]

Natural human serum IgG Fc glycan has a fucosylation level of between 80 and 92%; however, the *in vitro* expressed recombinant IgG has a relatively high fucosylation level of over 90%,[53] indicating that the fucosylation level could be a regulatory mechanism for antibody activity. While elevations in the serum levels of fucosylated IgG were observed in association with fetal maternal allotype thrombocytopenia, natural variations in the levels of fucosylated serum IgG in healthy or immune compromised conditions have not been reported to date.[56]

Terminal Galactosylation

Terminal galactosylated Fcs account for about 63% of all Fc glycoforms[23] and have been shown to affect Fc affinity for FcγRs. For example, hypergalactosylation of Fc terminal glycan has been shown to increase Fc affinity for FcγRIIIa

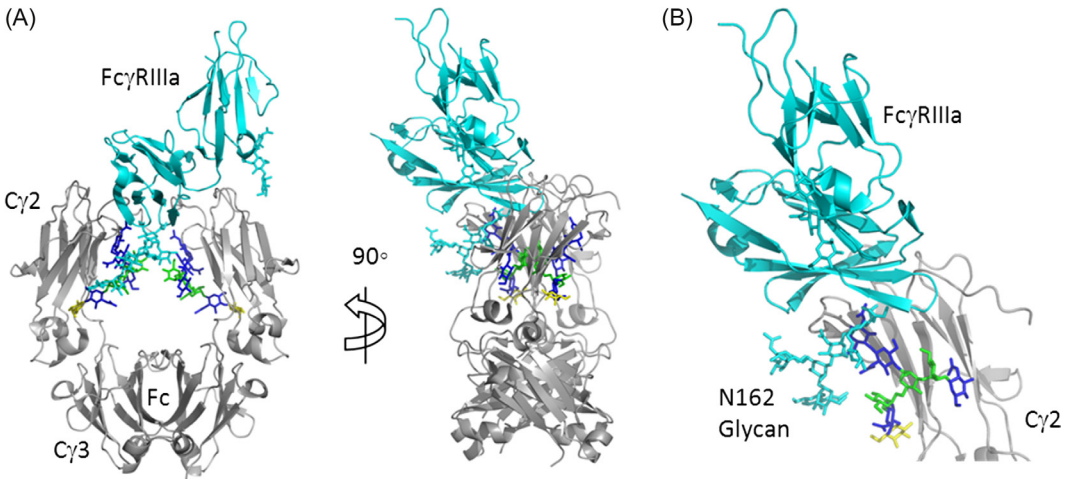

FIGURE 10.3 (A) Crystal structure of glycosylated FcγRIIIa in complex with afucosylated IgG_1 Fc (PDB 3AY4).[34,67] (B) Close–up view of glycan–glycan interactions. The receptor is shown in cyan and the IgG Fc is shown in gray. Glycans are shown as sticks, and the Fc glycans are colored according to Figure 10.2.

by threefold, presumably due to increased structural rigidity of the Fc Cγ2 domains.[57] In contrast, other reports suggest that hypogalactosylated Fc has increased affinity for FcγRs.[58] The level of Fc galactosylation has been shown to change with age,[59] and agalactosylation is found to be associated with inflammatory conditions such as rheumatoid arthritis.[58] Patients with rheumatoid arthritis have been found to have elevated levels of agalactosylated serum IgG glycans;[60] these patients showed an increase of 117% in serum agalactosylated IgG glycoform compared with the controls.[60] Further examination revealed that the IgG Fc is hypogalactosylated, whereas the Fab galactosylation level remained normal.[23] A similar study found that an increase in IgG agalactosyl glycoform is also present in a small number of other inflammatory diseases, including tuberculosis and Crohn's disease.[61] Interestingly, pregnant women affected by rheumatoid arthritis, who normally experience pregnancy-induced disease remission, show increased IgG galactosylation before reverting back to hypogalactosylation after giving birth.[62] The cellular mechanism behind this increased agalactosylated IgG level has been found to be due to lower galactosyltransferase activity in B cells;[63,64] however, this does not fully explain the selective agalactosylation of the Fc region. A suggested proinflammatory mechanism of rheumatoid arthritis, mediated by the agalactosylated glycoform, involves the binding of a cluster of agalactosylated Fc glycoform to the mannose-binding lectin, which subsequently activates the alternative complement pathway and leads to inflammation.[65] In contrast to its proinflammatory roles, a more recent study reveals an antiinflammatory pathway that requires Fc galactosylation. In this newly proposed antiinflammatory pathway, an immune complex formed by IgG_1 with a high level of Fc galactosylation promotes the association of inhibitory FcγRIIb and Dectin-1, a C-type lectin, and subsequently triggers an inhibitory signaling cascade.[66]

Terminal Sialylation

Terminal sialylation occurs in about 10% of Fc glycan[9,46] and, regardless of linkage type, has been shown to decrease Fc binding affinity for FcγRIIIa,[67] FcγRIIb, and the murine FcγRIIII by at least tenfold.[68] This decreased binding affinity correlates with decreased *in vivo* activities of hypersialylated monoclonal antibodies.[68] Although the crystal structure of sialylated Fc glycoform is unavailable, it has been speculated that the addition of a bulky sialic acid residue at the glycan terminus might impact the structure of the hinge that has been shown to affect FcγRs binding.[67,69] IgG Fc terminal glycan sialylation is regulated by the immune system. For example, active immunization was found to decrease the level of IgG Fc sialylation by 40%, an effect restricted to IgG but not IgM.[68] Moreover, a proinflammatory milieu during immunization favors the generation of antigen-specific IgG with lower Fc sialylation and *vice versa*.[70] However, this immunization-mediated decrease in antigen specific IgG Fc sialylation, observed in mouse models, does not seem to translate into human trials.[71] In addition to modulating Fc binding to FcγRs, Fc sialylation, in an α2,6-linkage-specific manner, has been shown to exert antiinflammatory properties in both mice and humans.[68,72,73] In fact, the α2,6-sialylated Fc fraction has been determined to be the active component of intravenous immunoglobulin (IVIg) therapy,[68] a common therapeutic used for treating a range of autoimmune conditions.[74] Moreover, recombinant hyper-α2,6-sialylated IgG Fc, at a tenfold-less dose, has been shown to recapitulate the therapeutic effect of IVIg in autoimmune mouse models.[72] On the other hand, this sialic acid-dependent antiinflammatory effect has not been reproducible in some mouse models.[75,76] This discrepancy could be explained by the different methods for enriching sialylated IVIg, or by the fact that different mouse strains react differently to IVIg treatment.

Terminal Mannose

High mannose glycoforms form a small fraction of total Fc glycoforms. High mannose glycoforms, including $Man_5GlcNAc_2$, generated using the GnTI-deficient HEK293S cell line, and $Man_9GlcNAc_2$, generated using the α-mannosidase inhibitor kifunesine, have been shown to increase Fc affinity for FcγRIIIa and decrease Fc affinity for FcγRIIb compared with Fc with biantennary complex glycans.[77,78] The increased affinity for FcγRIIIa is mainly attributed to the lack of core fucose at these oligomannose structures,[77] which is known to increase Fc affinity for FcγRIIIa.[49] In addition, $Man_9GlcNAc_2$ has been shown to perturb the quaternary organization of the Fc, but the impact of this on receptor binding has yet to be determined.[39] In addition to affecting FcγR binding, oligomannose glycoforms have been shown to increase Fc clearance rates,[79,80] possibly through mannose binding receptors on cell surfaces. On the other hand, some report the faster clearance of the oligomannose Fc to be due to the trimming of the oligomannose by serum mannosidases, an effect recapitulated by incubating oligomannose glycoform in human serum.[81] Studies using radiolabeled antibodies show that oligomannose glycoforms have clearance rates similar to those of other glycoforms; however, terminal mannose and terminal galactose increase the amount of Fc catabolized in the liver.[82]

Complement Activation

Fc glycosylation has been shown to be essential for effective binding to C1q and complement activation.[37,47,83] Different glycoforms exhibit different affinity for C1q and different efficacy in activating complement dependent cytotoxicity.[77] Specifically, complex type glycoforms, regardless of their fucosylation status, activate the complement cascade more efficiently than the hybrid or oligomannose glycoforms.[77] Moreover, increased terminal galactosylation has been found to increase C1q binding.[84] Furthermore, increased terminal sialylation and/or glycan flexibility might impair Fc affinity for C1q. The Fc glycan forms extensive hydrophobic and electrostatic interactions with the Fc protein backbone.[30,44,53] When this hydrophobic interaction is disrupted by mutating one of the hydrophobic residues, a significant increase in terminal galactosylation and sialylation was observed.[30,44] The increased terminal processing was accompanied by decreased Fc affinity for the C1q and consequently reduced Fc mediated complement activation.[30] However, generating the same hydrophobic F243A mutant in murine IgG_{2a} did not result in any difference in binding activity to C1q or complement activation compared to the wild type.[85] These contradictory results might be explained by the different IgG species and the different sialic acid species, Neu5Ac or Neu5Gc, present on Fc terminal glycan. Further investigation is needed to confirm the influence of increased glycan flexibility and terminal sialylation on Fc binding to C1q.

Besides directly influencing Fc binding to C1q, the Fc terminal GlcNAc has been shown *in vitro* to bind the mannose binding protein to indirectly activate the alternative complement cascade, a mechanism proposed to explain the inflammatory rheumatoid arthritis associated with decreased Fc terminal glycan galacosylation.[86,87] The physiological significance of this pathway is, however, less clear.

GLYCAN ENGINEERING OF ANTIBODIES

Given the importance of glycosylation for Fc function, the modification of *N*-linked glycosylation of IgG Fc has become a major focus for antibody engineering.[53,109] The most common mammalian expressions systems used to produce recombinant antibodies are the CHO, NS0,

and Sp2/0 cell lines. Glycosylation profiles of recombinant antibodies produced in these expression systems are characterized by predominantly fucosylated biantennary complex-type glycans similar to serum IgG. However, all of these cell lines show lower levels of galactosylation than serum IgG Fc.[88] NS0 and Sp2/0 cell lines are of murine origin and often contain glycans bearing α1→3-linked galactose residues that are unusual in humans and have led to hypersensitivity reactions in patients.[89]

These differences in glycosylation profiles of CHO, NS0, and Sp2/0 cell lines and more recent studies on enhanced effector functions of defucosylated antibodies have led to a greater focus on the production of recombinant antibodies with homogenous glycoforms.

Manipulations of the *N*-linked glycosylation pathway can yield unnatural Fc glycoforms with modified effector functions. Antibodies with oligomannose-type, hybrid-type, and defucosylated complex-type glycans have been shown to display differential binding to FcγRIIIa and varying levels of ADCC.[52,77] Defucosylation of antibodies has been associated with the greatest increase in ADCC. To date, no correlation between core fucosylation and increase in CDC has been reported; however, differences in terminal glycan residues of IgG Fc have been associated with differences in CDC activity. While mapping of amino acid residues on IgG Fc crucial for C1q binding has been reported,[83] there are currently no reported studies on the structural implications of IgG Fc glycosylation on Fc-C1q binding. Differences in ADCC and CDC activity of fucosylated and defucosylated IgG antibodies of all subclasses have again shown the potent effect of defucosylation on ADCC.[90] These differences in IgG Fc effector functions with variations in Fc glycosylation are summarized in Table 10.1. Reduced binding affinity to FcγRIIIa for terminal sialylated IgG Fc has also been reported previously;[67,68] however, no direct correlation between ADCC or CDC activity and Fc sialylation is known to date.

One of the first reported glycoengineered cell lines was generated to overexpress *N*-acetylglucosaminyltransferase III (GnT III), the Golgi enzyme responsible for the addition of a bisecting GlcNAc residue to the central β mannose of the Fc glycan. Recombinant

TABLE 10.1 Glycoforms of Human IgG Fc and Their Associated Effector Functions and Affinity for FcγRIIIa and C1q

IgG Fc Glycoform	FcγRIIIa (V158)	C1q	FcRn	ADCC	CDC
Complex type (+Fuc)	+++	+++	+++	+++	++++
Complex type (−Fuc)	++++++	+++	+++	+++++	++++
Hybrid type (+Fuc)	++	++	+++	+	++++
Hybrid type (−Fuc)	++++	+++	+++	+++++	++++
Degalactosylated (−Fuc)	++++	+++	nd	+++	nd
Degalactosylated (+Fuc)	+++	nd	nd	+	nd
Galactosylated (−Fuc)	++++	nd	nd	+++	nd
$Man_5GlcNAc_2$	++++	+	+	++++	+++
$Man_9GlcNAc_2$	+++++	++	++++	++++	+++

Source: Data based on Forthal et al.,[35] Kanda et al.,[54] Niwa et al.,[72] and Shinkawa et al.[93]
Note: Fucosylated and defucosylated Fc glycoforms are shown as +/− Fuc. Data not determined is shown as nd.

antibodies produced in this cell line displayed enhanced ADCC.[93] It was later discovered that the presence of bisecting GlcNAc residues inhibits core fucosylation and was responsible for the enhanced effector function.[92] Ferrara et al.[94] further engineered GnT III for improved localization and along with overexpression of Golgi α mannosidase II enzyme, produced recombinant antibodies with hybrid- or complex-type defucosylated glycans. This study led to the development of GA-101, a glycoengineered CD20 specific antibody that is now in Phase II clinical trials for non-Hodgkin lymphoma[95] and is being evaluated in Phase III studies in chronic lymphocytic leukemia. CHO cell lines lacking expression of α1→6 fucosyltransferase are also being used to produce defucosylated recombinant antibodies with enhanced ADCC function.[96] MEDI-563 is one such non-fucosylated antibody that has reached clinical testing.[97] Besides mammalian expression systems, extensively glyco-engineered strains of *Pichia pastoris*, *Lemna*, *Nicotiana benthamiana*, and moss have also been used to improve yields and lower the cost of production of recombinant antibodies.[52,53,98]

ENDOGLYCOSIDASES IN IMMUNE EVASION AND THERAPEUTICS

Removal of the *N*-linked glycan at Asn297 of the IgG Fc domains leads to loss of antibody effector functions and impaired complement activation.[99] Recombinant antibodies expressed in bacteria or designed with mutations at the Asn297 *N*-linked glycosylation site at the Fc domain are aglycosylated and unable to trigger ADCC and inflammation. Such aglycosylated antibodies are particularly suited in a therapeutic setting where the mode of action of the recombinant antibody is through high-affinity binding to target antigen and Fc-mediated effector functions are unnecessary or may be detrimental.

Deglycosylation of antibodies by bacterial endoglycosidases serves as an important strategy for immune evasion.[100,107] Endoglycosidases are a class of enzymes that cleave the GlcNAc β1→4GlcNAc linkage in the chitobiose core of *N*-linked glycans. EndoS is one such endoglycosidase secreted by the Gram-positive pathogenic bacteria *Streptococcus pyogenes*. It exhibits a surprising specificity for native glycosylated IgG.[100] EndoS can successfully hydrolyze *N*-linked glycans from all four subclasses of IgG and leads to loss of antibody effector functions and complement activation.[101]

This unique property has been further used to treat autoimmune disease phenotypes in animal models. EndoS treatment of mice models of systemic lupus erythematosus led to significantly prolonged life and reduced disease.[102] Further, treatment of healthy rabbits with low doses of EndoS led to no toxicity and minimal production of anti-EndoS antibodies. EndoS is currently being studied as a potential therapeutic for autoimmune diseases in humans.[103]

A further application of IgG-specific endoglycosidases is in the boosting of monoclonal antibody effector functions.[48] At physiological antibody concentrations, the FcγRs of effector cells are largely saturated by serum IgG. A consequence of this saturation is that large amounts of therapeutic antibody are required to achieve cell killing. It is also likely that monoclonal antibody therapies that rely on immune-recruitment through their Fc domains are only effective against high-copy antigens. The problem of competing serum IgG can be solved by the deglycosylation of serum IgG with EndoS, while the monoclonal antibody is engineered to be resistant to the hydrolase, for example, by expressing the antibody with bisecting GlcNAc or as hybrid- or oligomannose-type glycans.[104] This technique has a significant impact on receptor binding by the monoclonal antibody and may find utility in broadening the therapeutic impact of monoclonal antibodies.

VARIATIONS OF ANTIBODY GLYCOSYLATION IN DISEASE

Variations in the glycosylation profile of serum IgG have been correlated with age, pregnancy, and several inflammatory diseases.[59,62,105] Levels of agalactosylated and bisected GlcNAc-containing Fc glycans increase with age.[23,59,60] Similarly, sialylation of IgG decreases with age.[106] Elevated levels of agalactosylated Fc glycoforms have been observed in patients with rheumatoid arthritis.[60] Early studies suggested that agalactosylated antibodies may play a causative role in inflammatory disease pathology.[65,86] However, elevated levels of agalactosylated antibodies in several other inflammatory conditions have since been reported,[23] and it now appears that agalactosylation may in fact be a consequence of inflammation.

Terminal sialylation of IgG Fc has been shown to lead to potent antiinflammatory effects,[68] and sialylated IgG Fc has been shown to be the active antiinflammatory component of intravenous immunoglobulin therapy.[72] Specifically, IgG Fc containing terminal α2→6-linked sialic acid residues confers enhanced antiinflammatory activity in both mouse models of thrombocytopenia and *in vitro* ADCC assays.[72]

While the exact mechanism for this activity is still not completely clear,[14] recent evidence showed that mouse IgG Fc with sialylated Fc binds to SIGNR1, a mouse homolog of the human dendritic cell lectin DC-SIGN. Blockade of Fc-SIGNR1 binding or genetic deletion of SIGNR1 expression led to loss of antiinflammatory properties of sialylated Fc.[13]

CONCLUSION AND PERSPECTIVES

Antibody glycosylation has important consequences on antibody effector functions both within the natural repertoire of immunoglobulin glycoforms and on antibody-based therapeutics. In the case of IVIg therapy, understanding the mode of action on the molecular level and the role of glycosylation will guide the development of recombinant alternatives to the human-derived material in current use and may provide routes to increased efficacy. Engineering glycosylation is a well-established route to enhance the *in vivo* efficacy of many therapeutic monoclonal antibodies. Exploration of the selective deglycosylation of competing serum IgG offers a route to the further enhancement of this important class of therapeutic agent.

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