Antibody Glycosylation and Its Impact on the Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies and Fc-Fusion Proteins

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ABSTRACT: Understanding the impact of glycosylation and keeping a close control on glycosylation of product candidates are required for both novel and biosimilar monoclonal antibodies (mAbs) and Fc-fusion protein development to ensure proper safety and efficacy profiles. Most therapeutic mAbs are of IgG class and contain a glycosylation site in the Fc region at amino acid position 297 and, in some cases, in the Fab region. For Fc-fusion proteins, glycosylation also frequently occurs in the fusion partners. Depending on the expression host, glycosylation patterns in mAb or Fc-fusions can be significantly different, thus significantly impacting the pharmacokinetics (PK) and pharmacodynamics (PD) of mAbs. Glycans that have a major impact on PK and PD of mAb or Fc-fusion proteins include mannose, sialic acids, fucose (Fuc), and galactose (Gal). Mannosylated glycans can impact the PK of the molecule, leading to reduced exposure and potentially lower efficacy. The level of sialic acid, N-acetylneuraminic acid (NANA), can also have a significant impact on the PK of Fc-fusion molecules. Core Fuc in the glycan structure reduces IgG antibody binding to IgG Fc receptor IIIa relative to IgG lacking Fuc, resulting in decreased antibody-dependent cell-mediated cytotoxicity (ADCC) activities. Glycoengineered Chinese hamster ovary (CHO) expression systems can produce afucosylated mAbs that have increased ADCC activities. Terminal Gal in a mAb is important in the complement-dependent cytotoxicity (CDC) in that lower levels of Gal reduce CDC activity. Glycans can also have impacts on the safety of mAb. mAbs produced in murine myeloma cells such as NS0 and SP2/0 contain glycans such as Galα1–3Galβ1–4N-acetylglucosamine-R and N-glycolylneuraminic acid (NGNA) that are not naturally present in humans and can be immunogenic when used as therapeutics.
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INTRODUCTION

The ground-breaking discovery of monoclonal antibody (mAb) technology by Kohler and Milstein in 1975[1] provided the possibility of creating antibodies as a class of therapeutics. The promises of mAb drugs, however, were not realized initially because of the hurdles of immunogenicity issues associated with early therapeutic antibodies being murine origin. Murine antibodies generally induce anti-drug antibody (ADA) responses in humans, potentially resulting in severe adverse effects. Orthoclone OKT3 anti-CD3 antibody was the first mAb approved in 1986 for human use for transplantation rejection.[2] Being of murine origin, OKT3 induced cytokine release syndrome and caused accelerated clearance of the drug because of human anti-murine antibody (HAMA) responses.[3,4] The real promise of mAb as a class of blockbuster therapeutics was realized a little more than a decade later when a murine–human chimeric mAb against CD20, rituximab, was approved for the treatment of non-Hodgkin lymphoma (NHL) in 1997.[5] The breakthrough was made possible because of advances in molecular biology, genetics, protein engineering, cell sciences, and in applied sciences from the biotechnology and pharmaceutical industry. The revolution in molecular biology in the 1980s made humanization of murine antibody possible and, eventually, a fully humanized antibody was successfully developed.[6] Humanization greatly reduces a therapeutic antibody's immunogenicity in humans, making chronic administration possible. Such advance in antibody technologies has resulted in the explosive development of therapeutic mAbs over the last decade. Today, more than 40 mAbs and derivative drugs have been approved for human use with many of them attaining blockbuster status.[7] It is estimated that in the near future, about 30% of the new drugs will be antibodies or antibody derivatives[6]. Antibody derivatives include Fc-fusion proteins, antibody–drug conjugates (ADCs), immunocytokines (antibody–cytokine fusion) and antibody–enzyme fusions. The approved mAb and antibody-derived drugs cover many therapeutic areas with most products in the oncology and autoimmune/allergy/inflammation area.[7] Intact whole mAb and ADC therapeutics are mostly in the oncology area, whereas current

Abbreviations used: ADA, anti-drug antibody; ADC, antibody drug conjugate; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; ASGPR, asialoglycoprotein receptor; Asn, asparagine; AUC, area under curve; BR3, B cell-activating factor receptor 3; CCR4, C-C chemokine receptor type 4; CDC, complement-dependent cytotoxicity; CHO, Chinese hamster ovary; CLL, chronic lymphocytic leukemia; C_{max}, maximum concentration; CTLA4, cytotoxic T lymphocyte-associated protein 4; EGFR, epidermal growth factor receptor; Fc, fragment crystallizable; FcRn, neonatal Fc receptor; FcγR, IgG Fc receptor; Fuc, fucose; Fv, fragment variable; Gal, galactose; GlcNAc, N-acetylglucosamine; GnT, N-acetylglucosaminyltransferase; GalNAc, N-acetylgalactosamine; HAMA, human anti-murine antibody; LFA3, lymphocyte function-associated molecule 3; mAb, monoclonal antibody; ManR, mannose receptor; NANA, N-acetylneuraminic acid; NGNA, N-glycolylneuraminic acid; NHL, non-Hodgkin lymphoma; PD, pharmacodynamics; PK, pharmacokinetics; TNFRII, tumor necrosis factor α receptor II.

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Fc-fusion proteins are mostly used for the treatment of autoimmune diseases and for enzyme replacement therapy. Tables 1 and 2 list all antibody therapeutics and Fc-fusion proteins approved for marketing as of November, 2014.

Like natural IgGs, all approved recombinant therapeutic mAbs are glycosylated, although some nonglycosylated mAbs or derivatives are in clinical development.[8] Therapeutic mAbs or derivatives have an asparagine (Asn)-X-Ser/Thr (where X is any amino acid except a Pro) consensus sequence for N-glycosylation at the position Asn^{297} in the heavy chain of the CH2 constant domain. Some therapeutic mAbs also bear additional glycosylation in the Fab domain such as cetuximab at Asn^{88} of the VH region.[9] In addition, some of the Fc-fusion partner molecules such as etanercept and B cell-activating factor receptor 3 (BR3)–Fc also possess O-linked glycans.[10,11] Alteration of glycan compositions and structures can cause conformational changes of the Fc domain, which could change binding affinity to Fcγ receptors, resulting in changes of immune effector functions.[12,13] Fc effector functions include complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cell-mediated phagocytosis (ADCP). All three effector functions are triggered by the formation of immune complexes, which then recruit complement proteins and/or effector cells. IgG Fc receptors (FcγRs) are a family of molecules consisting of three activating (FcγRI, FcγRIII, and FcγRIV in mice; FcγRIa, FcγRIIa, and FcγRIIIa in humans) and one inhibitory (FcγRIIb) receptor.[14] It is well established that the absence of glycosylation dramatically reduces the binding affinity to FcγRI and eliminates the binding to FcγRII and FcγRIII receptors.[15,16] Alteration of some glycoforms in therapeutic mAb or Fc-fusions can also impact the pharmacokinetics (PK) of the molecules,[17,18] exemplified by the negative impact on the PK of high-mannose glycans, presumably mediated by C-type lectin clearance mechanisms. In the last decade, biopharmaceutical companies have put tremendous efforts into the development of mAbs with higher effector functions (biobetters). The best example of such a biobetter is Gazyva® (obinutuzumab) developed by Roche/Glycart, which is the third-generation type II anti-CD20 humanized and glycoengineered mAb with dramatically enhanced ADCC activity.[19] Gazyva® demonstrated superior efficacy over Rituxan® (rituximab) in clinical trials for the treatment of chronic lymphocytic leukemia (CLL), leading to US FDA approval in November, 2013.[20]

The importance of biologics drugs in disease management and the high cost of such drugs have prompted the development of biosimilars. Guidelines for biosimilar drug development from several regulatory agencies across the world including FDA and EMEA consider quality attributes especially glycosylation similarities as one of the most critical requirements.[21–26] Understanding the impact of glycosylation on PK and pharmacodynamics (PD) and ensuring tight control of glycosylation during manufacturing are required for both novel and biosimilar mAb and Fc-fusion drug development. In this review article, background information related to glycosylation compositions and patterns (glycoforms) of therapeutic mAb or Fc-fusion proteins are reviewed and the impact on PK and PD is discussed. The impact of glycosylation on ADC generally follows the rules of intact mAb. However, additional considerations in conjugated drug and positions of conjugation are also important in relation to PK and PD, but will not be discussed here.

IgG GLYCAN STRUCTURES AND BIOSYNTHESIS

Unlike other serum proteins, the N-linked glycans of human IgGs are typically biantennary complex structures (Fig. 1). A conserved core structure is composed of two N-acetylglucosamine (GlcNAc), three mannose, and two GlcNAc residues that are β-1,2 linked to α-6 mannose and α-3 mannose, forming two antennae. Additional monosaccharides extended from the core may be present. Depending on the host glycosylation machinery, additional fucose (Fuc), galactose (Gal), bisecting GlcNAc, and sialic acid including N-acetylneuraminic acid (NANA) or N-glycolylneuraminic acid (NGNA) residues, may be added to the core. Glycans present considerable heterogeneity with more than several hundred possible glycoforms because of the random pairing of heavy-chain glycans with different structures.[9,27] The α-1,6 arm of the biantennary glycan extends along the hydrophobic face of the CH2 amino acid backbone where the polar nature of the carbohydrate protects the underlying hydrophobic polypeptide. The α-1,3 arm of the glycan extends toward the interstitial space formed by the CH2–CH3 dimer. The glycans on opposite Asn^{297} residues of each heavy chain interact and maintain the conformation of the Fc domain. As such, changes in Fc glycosylation can alter the Fc conformation and subsequent binding to Fc receptors.[28]

Antibody N-linked glycan biosynthesis follows the same process as for other glycoproteins.[29–31] A preassembled Glc3Man9GlcNAc2 oligosaccharide is transferred to Asn^{297} of the IgG heavy chain by an oligosaccharyltransferase complex in the endoplasmic reticulum, followed by glycosidase-mediated sequential removal of three Glc residues and a mannose residue to form Man8GlcNAc2. After transition to the *cis*-Golgi apparatus, three additional mannoses are trimmed by mannosidase to form a Man5GlcNAc2 oligosaccharide. Once in the medial Golgi, the N-acetylglucosaminyltransferase–I (GnT-I) enzyme mediates the transfer of GlcNAc from UDP-GlcNAc to the O-2 position of the terminal mannose residue in the α1 → 3 branch of the Man5GlcNAc2 oligosaccharide. Subsequently, removal of two more mannose residues from the α1→6 branch gives rise to a GlcNAcMan3GlcNAc2 oligosaccharide. Before translocating to the *trans*-Golgi, N-acetylglucosaminyltransferase–II (GnT-II) mediates the transfer of an additional GlcNAc to the newly generated terminal mannose residue at the O-2 position in the α1→6 branch to form the conserved biantennary core structure GlcNAc2Man3GlcNAc2.[31] In the *trans*-Golgi, the addition of Gal, bisecting GlcNAc, sialic acid (either NANA or NGNA) and core Fuc residues occurs via enzymatic addition by specific transferases using nucleotide-charged monosaccharide donors. Figure 2 summarizes the major steps of N-linked glycan biosynthesis. Familiarization with the steps of glycan biosynthesis may help in the understanding of the specific glycosylation patterns associated with effector functions. For instance, high mannose variants (Man5/8/9) would also lack core Fuc, resulting in higher binding affinity to FcγRIIIa and enhanced ADCC activity (see section *Glycosylation Impact on PD of mAb*).

O-linked glycosylation does not occur in IgG molecules, but is present in many membrane proteins such as TNFαRII in Fc-fusion molecule etanercept and in human mucin and IgA1.[32,33] O-glycosylation is fundamentally different from N glycosylation as a typical consensus amino acid sequence has not been clearly identified yet.[34] There are several types of O-linked

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Table 1. Monoclonal Antibodies Approved for Marketing as of November, 2014

Mechanism of Action	Brand Name	INN	Target	IgG Subclass	First Approved Indications	Therapeutic Area
Check point inhibitors (immunotherapy)	Yervoy	Ipilimumab	CTLA-4	Human IgG1	Advanced melanoma	Oncology
	Keytruda	Keytruda	PD-1	Humanized gG4	Ipi refractory melanoma	Oncology
	Opdivo	Nivolumab	PD-1	Human IgG4	Advanced melanoma (Japan)	Oncology
Targeted therapy involving ADCC, CDC, and other mechanisms	Rituxan	Rituximab	CD20	Chimeric IgG1	Non-Hodgkin lymphoma	Oncology
	Herceptin	Trastuzumab	Her2	Humanized IgG1	Her2-positive breast cancer	Oncology
	Avastin	Bevacizumab	VEGF	Humanized IgG1	Coloerectal, NSCLC-squamous, renal cancer	Oncology
	Erbitux	Cetuximab	EGFR	Chimeric IgG1	Head and neck, kras-ve colorectal cancer	Oncology
	Campath	Alemtuzumab	CD52	Human IgG1	B-cell chronic lymphocytic leukemia	Oncology
	Vectibix	Panitumumab	EGFR	Human IgG2	Metastatic colorectal carcinoma	Oncology
	Arzerra	Ofatumumab	CD20	Human IgG1	B-cell chronic lymphocytic leukemia	Oncology
	Perjeta	Pertuzumab	HER2	Humanized IgG1	Her2-positive breast cancer	Oncology
	Cyramza	Ramucirumab	VEGFR2	Human IgG1	Stomach cancer	Oncology
Antibody–drug conjugate	Kadcyla	Trastuzumab emtansine	HER2	Humanized IgG1	Her2-positive breast cancer	Oncology
	Adcetris	Brentuximab vedotin	CD30	Chimeric IgG1	Refractory HL, refractory relapse sALCL	Oncology
	Mylotarg	Gemtuzumab ozogamicin	CD33	Humanized IgG1	Acute myeloid leukemia (withdrawn)	Oncology
Targeted radiotherapy	Bexxar	Tositumomab-iiodine-131	CD20	Murine IgG2a	Non-Hodgkin lymphoma	Oncology
	Zevalin	Ibritumomab-tiuxetan Y90 or In111	CD20	Murine IgG1	Non-Hodgkin lymphoma	Oncology
Inflammatory cytokine inhibitors	Remicade	Infliximab	TNF-α	Chimeric IgG1	Rheumatoid arthritis, Crohn's diseases	Immunology
	Simponi	Golimumab	TNF-α	Human IgG1	Rheumatoid arthritis, Crohn's diseases	Immunology
	Humira	Adalimumab	TNF-α	Human IgG1	Rheumatoid arthritis, Crohn's diseases	Immunology
	Cimzia	Certolizumab	TNF-α	Humanized IgG1[a,b]	Crohn's diseases, rheumatoid arthritis	Immunology
	Stelara	Ustekinumab	IL-12/IL-23	Human IgG1	Plaque psoriasis	Immunology
	Ilaris	Canakinumab	IL-1β	Human IgG1	Cryopyrin-associated periodic syndromes	Immunology
	Benlysta	Belimumab	Blys	Human IgG1	Systemic lupus erythematosus	Immunology

Continued

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Table 1. Continued

Mechanism of Action	Brand Name	INN	Target	IgG Subclass	First Approved Indications	Therapeutic Area
Receptor blockade	Actemra/roactemra	Tocilizumab	IL-6R	Humanized IgG1	Rheumatoid arthritis	Immunology
	Simulect	Basiliximab	IL-2Rα	Chimeric IgG1	Transplantation rejection	Immunology
	Othorclone OKT3	Muromonab OKT3	CD3	Murine IgG2a	Transplantation rejection	Immunology
IgE inhibitor	Xolair	Omalizumab	IgE	Humanized IgG1	Asthma	Immunology
Integrin inhibitor	Entyvio	Vedolizumab	α4β7	Humanized IgG1	Ulcerative colitis and Crohn's disease	Immunology
	Tysabri	Natalizumab	α4	Humanized IgG4	Multiple sclerosis	Immunology
Anti-infection	Synagis	Palivizumab	RSV F	Humanized IgG1	RSV infection	Infection
	Abthrax	Raxibacumab	Anthrax toxin	Human IgG1	Anthrax infection	Infection
Bone regeneration	Prolia/Xgeva	Denosumab	RANKL	Human IgG2	Osteporosis	Bone disease
Antiplatelets	Reopro	Abciximab	GPIIb/IIIa	Chimeric IgG1[a]	Ischemia/angina	Cardiovascular
Complement inhibitor	Soliris	Eculizumab	C5	Humanized IgG2/4	Paroxysmal nocturnal hemoglobinuria	Hematology
Anti-VEGF	Lucentis[a]	Ranibizumab	VEGF	Humanized IgG1[a]	Age-related macular degeneration	Ophthalmology

[a]Fab fragment.
[b]PEGylated.
INN, international nonproprietary name.

glycosylations and the mucin-type O-glycan, with N-acetylgalactosamine (GalNAc) at the reducing end, is the most common form in secreted and plasma membrane proteins in humans.[35] O-glycan biosynthesis occurs in a stepwise fashion involving the sequential transfer of single sugar residues by distinct glycosyltransferases. The initiation of a mucin-type O-glycan formation involves the transfer of a GalNAc residue from the nucleotide sugar UDP-GalNAc to the hydroxyl side chains of Ser/Thr and is catalyzed by a family of more than 20 different polypeptide GalNAc transferases.[34,35] These carbohydrates can be further elongated by incorporation of Gal, Fuc, GlcNAc, and sialic acid residues via different linkages.[36] Figure 3 depicts the mucin type O-glycan core structure.

GLYCOSYLATION DIFFERENCES BY DIFFERENT EXPRESSION HOSTS

It has been well demonstrated that different host cells can add different glycoforms to recombinant antibodies.[6,37–39] Most approved mAbs are produced in Chinese hamster ovary (CHO) cells and some are produced in murine myeloma cell lines NS0 and SP2/0. The Fc glycans of human serum IgG are mainly of a core fucosylated complex biantennary type with predominantly G0F, G1F, and G2F glycoforms and trace amounts of the sialylated glycoforms.[9] In addition, a small percentage of human IgG with afucosylated glycans with or without a bisecting GlcNAc residue also exist. Recombinant human IgG made in CHO cells has similar glycosylation patterns or glycoforms to naturally occurring human serum IgG.[9,38] Murine myeloma cells (NS0 and SP2/0) can add sugars that are not naturally found on normal human IgG and are known to be immunogenic, for example, α-Gal epitope (Galα1–3Galβ1–4GlcNAc-R) and NGNA.[9,39] NGNA is the predominant sialic acid in the glycoproteins produced in murine cells and only trace amount found in CHO-produced glycoproteins.[6,37] Nonhuman glycoforms produced by murine expression hosts may present important implications in terms of immunogenicity of therapeutic mAbs (see section *Glycosylation Impact on Immunogenicity of Therapeutic mAb*).

The rat myeloma cell line YB2/0 produces recombinant mAb with low Fuc content and could elicit up to 50-fold higher ADCC activity than when produced in CHO cell.[40] Glycoprotein synthesized in wild-type yeast is afucosylated and bears high-mannose oligosaccharides with the number of mannose residues varying between individual strains.[41,42] *Pichia pastoris* has been successfully engineered to produce specific homogeneous human glycoforms on human IgG-Fc.[43] Production of therapeutic mAb in plants may offer an economic solution to the high cost of mAb drugs. Significant achievements in humanizing glycosylation and removal of enzymatic pathways that generate immunogenic residues on glycoproteins in plant have been reported.[44] A plant-derived mAb has yet to reach the market, although Elelyso (taliglucerase alfa), a recombinant enzyme made in plant to treat Gaucher disease, has been approved by the FDA in 2012.[45] It was stated in the product monograph[46] that some Elelyso-treated patients developed ADA. However, it is not clear whether the ADA rate is higher than that of the CHO-derived product Cerezyme (imiglucerase) or whether it is glycosylation related.[46,47] More recently, recombinant mAbs against the Ebola virus produced in tobaccos plant *Nicotiana benthamiana* made headlines around the globe. Here, it was claimed that a plant produced mAb cocktail

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Table 2. Fc-Fusion Proteins Approved for Marketing as of November, 2014

Trade Name	INN	Target/MOA	Glycosylation	Fusion Partner	Indications
Enbrel	Etanercept	TNF-α	Y	TNFα-IIR	Rheumatoid arthritis
Amevive	Alefacept	CD2	Y	LFA-3	Plaque psoriasis
Orencia	Abatacept	CD28	Y	CTLA-4	Rheumatoid arthritis
Arcalyst	Rilonacept	IL-1	Y	IL-1R	Plaque psoriasis
Nplate	Romiplostim	TPOR	N	TPO peptide mimetic	Chronic ITP
Nulojix	Belatacept	CD28	Y	CTLA-4 (L104E, A29Y)	Transplantation rejection/prophylaxis
Eylea	Aflibercept	VEGF-A and PGF	Y	VEGFR1/2	Macular degeneration (AMD, also ARMD)
Alprolix	FIX-Fc fusion	Coagulation	Y	FIX	Hemophilia B
Eloctate	FVIII-Fc fusion	Coagulation	Y	FVIII	Hemophilia A

INN, international nonproprietary name; ITP, idiopathic thrombocytopenic purpura.

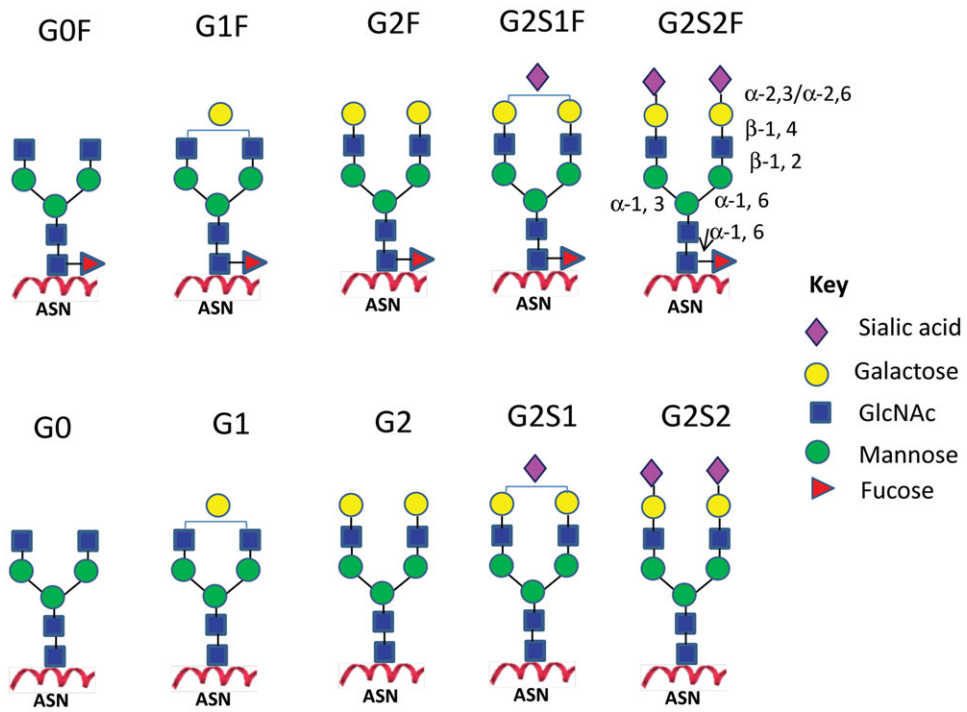

Figure 1. Major N-linked glycoforms of mAb therapeutics.

may have contributed to the successful recovery of two US aid workers who had contracted the Ebola virus.[48] This cocktail of mAbs possesses predominantly the afucosylated G0 glycoform.[49]

Although some marketed mAb drugs are produced in NS0 or SP2/0 murine myeloma cell lines, the glycosylation pattern in general are similar (except in the cases of sialyation and α-Gal epitope addition) to those produced in CHO cells. A market surveillance study was conducted to compare glycosylation profiles of marketed mAb drugs in the Swiss market. The results of the survey showed that product lot-to-lot variability was found to be generally low, suggesting that a majority of manufacturers have high-quality standards in their production processes. However, proportions of G0, G1, and G2 core-fucosylated chains derived from different products varied considerably and showed a bias toward the immature agalactosylated G0 form.[50] From the 16 mAb drugs tested, the predominant glycoform is G0F (30%–99%), followed by G1F (1–6 linkage, 1%–35%), G1F (1–3 linkage, 0%–10%), and G2F (0%–30%).[50]

FcγRs AND RELATED BIOLOGICAL FUNCTIONS

The Fab and Fc domain of an IgG antibody have distinct functions in the immune system, linking the innate arm to the adaptive arm of immunity.[51] Antibodies bind through the Fab domain to specific antigens and through the Fc domain to Fc receptors on multiple cell types to initiate a cascade of various immune responses. In humans, there are four types of Fc receptors for IgG molecules: FcγRI (also known as CD64), RII (CD32 a, b, and c), RIII (CD16 a, b, and c), and neonatal Fc receptor (FcRn). These receptors are expressed by many cells including both immune cells and nonimmune cells.[51,52] Antibody engagement of activating type Fc receptors, which include FcγRI, FcγRIIa, and IIc, FcγRIIIa, triggers signaling through an immunotyrosine-like activation motif and induces a cell-based inflammatory responses including ADCC, CDC, and ADCP. Signaling through an immunotyrosine-like inhibitory motif present in FcγRIIb activates an intracellular phosphatase, thereby dampening an inflammatory response.[28]

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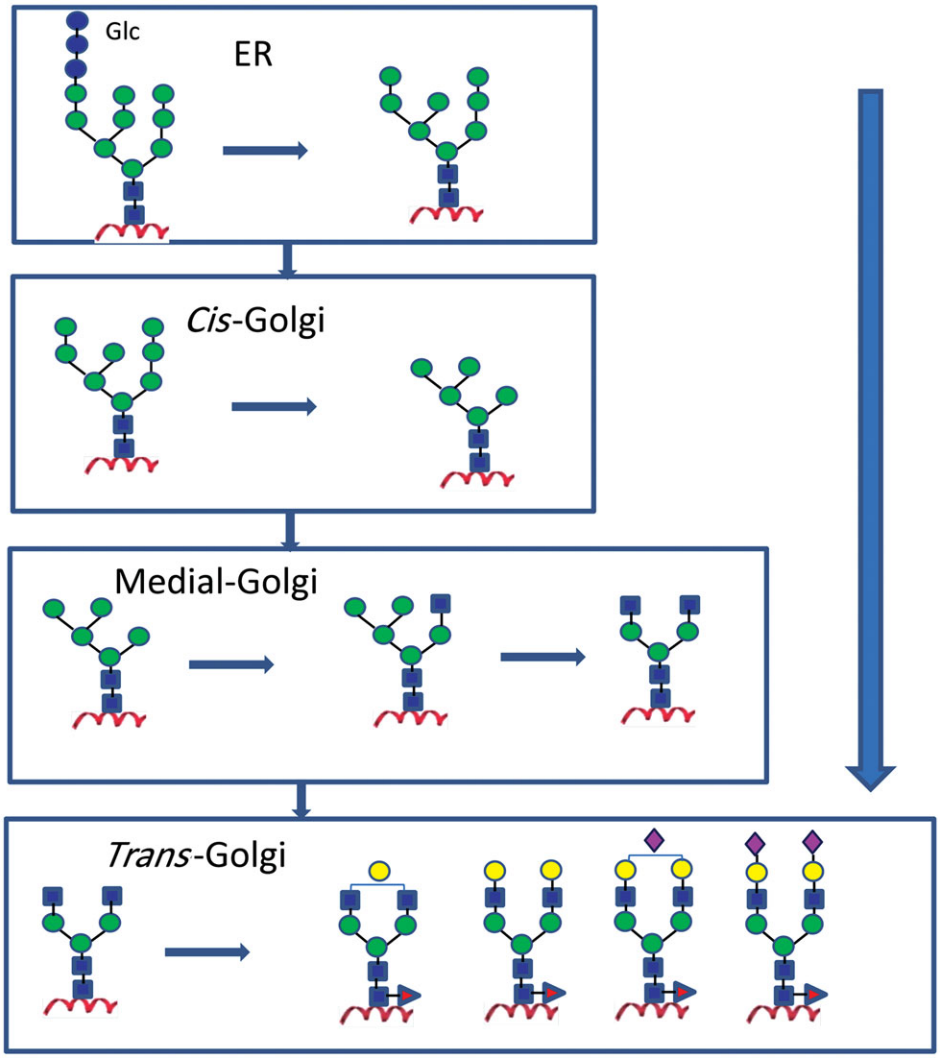

Figure 2. Antibody N-linked glycan biosynthesis pathways. Schematic presentation is based on the work of Kornfeld and Kornfeld.[31]

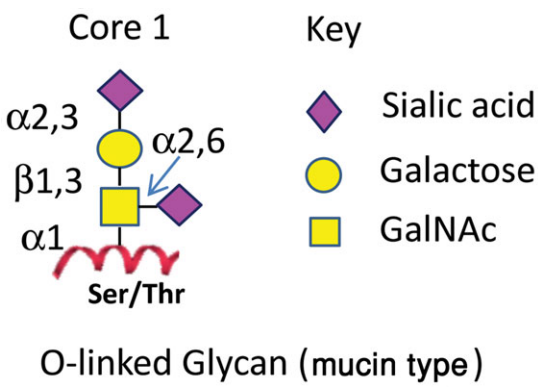

Figure 3. O-linked glycans.

FcγRI is a high-affinity receptor, whereas FcγRII and FcγRIII are low-affinity receptors for monomeric IgG molecules. The FcγRI family is the only Fcγ receptor class capable of appreciably binding monomeric IgG *in vivo*.[53] The so-called low-affinity FcγRs bind monomeric antibodies poorly. However, they can bind antibodies as multivalent immune complexes with high avidity. As a consequence, in the absence of antigen, low-affinity FcγRs remain free in spite of the high concentration of circulating immunoglobulins, (e.g., IgG). They are therefore more suitable than high-affinity FcγR to enable antibodies to modulate an immune complex-driven adaptive immune response.[53] Because affinity of human IgG to hFcγR is dependent on whether it is in the monomeric or immune complexed state, care needs to be taken when comparing the binding affinities. The biology of FcγRIII has been investigated more extensively than that of other Fcγ receptors, possibly because of the role of activating FcγRIIIa in NK cell activity and ADCC, which both are closely related to autoimmunity and mechanism of action for cancer therapy by therapeutic mAbs.[28] FcγRIIIa has a discrete distribution across the hematopoietic system, and is found on cells such as monocytes and NK cells.[54] Recently, it was shown at the molecular level that a unique carbohydrate–carbohydrate interface was involved in the interaction between afucosylated IgG1 and FcγRIIIa, which explains the increased affinity for the afucosylated antibody through lack of steric hindrance, which is present for fucosylated forms.[55]

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Table 3. Relative Binding Affinity of Human IgG Subclasses to Human Fc Receptors

		Monomeric IgG				Immune Complex			
Receptors	Expression	IgG1	IgG2	IgG3	IgG4	IgG1	IgG2	IgG3	IgG4
FcγRI	Monocytes, macrophages	+	−	++	++	+++	−	+++	+++
FcγRIIa$_{H131}$	Monocytes, macrophages, dendritic cells neutrophiles, platelets, eosinophiles	−	−	−	−	+++	++	+++	++
FcγRIIa$_{R131}$	Same as FcγRIIaR131	−	−	−	−	+++	+	+++	+
FcγRIIb	B cells, monocytes, macrophages, dendritic cells, neutrophiles	−	−	−	−	+	−	++	+
FcγRIIc	NK cells, B cells	−	−	−	−	+	−	++	+
FcγRIIIa$_{F158}$	NK cells, monocytes	−	−	+/−	−	++	−	+++	++
FcγRIIIa$_{V158}$	NK cells, monocytes	−	−	++		+++	+	+++	++
FcγRIIIbNA1	Neutrophiles, eosinophiles	−	−	−	−	+++	−	+++	−
FcγRIIIbNA2	Neutrophiles, eosinophiles	−	−	−	−	+++	−	+++	−
FcγRIIIbSH	Neutrophiles, eosinophiles	−	−	−	−	+++	−	+++	−
FcRn	Cells of endothelial/epitherial origin, monocyte, dendritic cells, kidney podocytes	+++	+++	++	+++	ND	ND	ND	ND

Adapted from Bruhns et al.[53]; Jefferis and Lund[52]; Roopenian and Akilesh.[56]
ND, no data available.

Currently, all approved therapeutic mAbs and their derivatives are of three human IgG: IgG1, IgG2, and IgG4. The major differences between these IgGs are the length of the hinge region, amino acid composition, and the numbers of disulfide bridges between the two heavy chains, which can have implications in terms of FcγR binding, effector functions, and PK properties. A canonical glycosylation site is located in the Fc region cγ2 domain Asn^{297} in all IgG subclasses.[13] Most of the currently approved IgG are IgG1 (~75%), although some IgG4 and IgG2 versions of IgG have been approved with more in development (Table 1). The lack of use of IgG3 may be because of its unfavorable PK in that IgG1, 2, and 4 have a terminal half-life around 21 days, whereas the half-life of IgG3 is only about 7 days. IgG subclasses have differential binding affinity to the IgG Fcγ receptors and consequently mediate variable levels of effector function (e.g., ADCC, CDC, and ADCP). The binding specificity and affinity of human IgG subclasses to human FcγRs have been studied extensively with generally similar results cross the studies, although some minor differences have been reported.[28] In a comprehensive study of human IgG subclass binding to human FcγRs, Bruhns et al.[53] showed that: (1) in monomeric form, IgG1 and IgG4 exhibited bindings to FcγRI, but not to any other human FcγRs, whereas IgG2 did not bind to any human FcγRs; IgG3 showed binding to FcγRI and FcγRIIIa$_{V158}$ but not to other FcγRs; (2) in immune complex form, IgG1 and IgG3 exhibited strong binding to most of the human FcγRs; IgG2 bound to FcγRIIa$_{H131}$, FcγRIIa$_{R131}$, and FcγRIIIa$_{V158}$ with various affinities; IgG4 bound to FcγRI, FcγRIIa, IIb, and IIc, and FcγRIIIa$_{V158}$; (3) the inhibitory receptor FcγRIIb had a lower affinity for IgG1, IgG2, and IgG3 than all other hFcγRs. The effector functions from antibody–Fc receptor engagement often determine the choices of IgG subclass used in therapeutic interventions for particular disease indications. For instance, IgG1 is often used in the indications that require Fc effector functions, whereas IgG4 or IgG2 is used where the effector function is not required or desired. Table 3 summarizes the relative binding affinity of different IgG subclasses in monomeric or immune complex form to Fcγ receptors and the cellular expression of FcγRs.

The FcRn is different from the other Fc receptors and is more similar in structure to MHC class I. The mature FcRn receptor consists of a complex of two subunits: p51 (α chain) and p14 (β2-microglobulin) and forms an MHC class-l-like heterodimer.[56–58] FcRn binds to the Fc portion of IgG at CH2–CH3 hinge region that is distinct from the binding sites of the classical FcγRs or the C1q component of complement, and it is the same versatile region of Fc that also binds staphylococcal protein A and streptococcal protein G, and rheumatoid factor.[56] The receptor is located mostly intracellulaly in numerous tissues and cells, such as the vascular endothelium, placenta, and myeloid derived antigen-presenting cells, such as monocytes, macrophages, and dendritic cells[56–62] (Table 3). The key function of FcRn is to salvage IgG and albumin in a pH-dependent manner to regulate the serum half-life of these molecules and maintain their homeostasis.[56,63–66] Specifically, an IgG or albumin molecule is either endocytosed or pinocytosed into the cells and sorted to the acidic endosomes where it binds FcRn. The FcRn-bound IgG or albumin is transported to the cell surface and released at the neutral pH of blood, thereby preventing it from undergoing lysosomal degradation, leading to its long half-life.[56] FcRn also functions in the transfer of IgG from mother to fetus[67,68] and is involved in binding IgG immune complexes for antigen presentation.[69–71]

IMPACT OF GLYCOSYLATION ON THE PK of mAb and Fc-FUSION PROTEINS

Glycosylation Is Not Required for IgG Antibody's Long Half-Life

It is well known that the removal of glycans from IgG can change the conformation of the Fc domain.[12,72–74] Most studies have shown that CH2 domain of deglycosylated IgG exhibited the "closed" confirmation, in contrast to the "open" confirmation of the glycosylated version.[12,75] However, a recent study by Borrok et al.[72] demonstrated that in solution state, aglycosylated Fc actually has a larger radius of gyration (Rg) than glycosylated Fc as it is in a more "open" form, suggesting that the proposed "closed" conformations of aglycosylated Fc observed in crystals may not be physiologically relevant. With a panel

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of comprehensive biophysical analytical tools, Alsenaidy et al. examined the physical stability of deglycosylated IgG1 mAb (by PNGase) and aglycosylated IgG1 Fc variants (made recombinantly) in comparison to the glycosylated versions.[73,74] They found relatively minor conformational changes of deglycosylated IgG1 mAbs or aglycosylated IgG1 Fc in less extreme conditions such as at lower temperature and near neutral pH, whereas significant conformational changes were observed in more extreme conditions such as at acidic pH and higher temperatures.[73,74]

However, the conformational changes caused by glycan removal do not appear to provide a meaningful impact on the PK properties of IgG *in vivo*. Nose and Wigzell[76] investigated the impacts of glycosylation with tunicamycin as a glycan depleting tool, and found that following i.v. administration, the serum concentrations of the deglycosylated mouse IgG2b antibodies were very similar to those of the glycosylated forms. Similar results were obtained by others with genetically engineered IgG mAbs.[15,77,78] For example, with genetically engineered mouse–human chimeric IgG1 and IgG3 antibodies, Tao and Morrison[15] demonstrated that the PK profile of aglycosylated IgG1 mAb with Asn^{297} mutation was almost identical to that of the glycosylated form, although aglycosylated IgG3 molecules showed relatively faster clearance. The reason for the shorter half-life of aglycosylated IgG3 remains unclear.

The clinical evidence for aglycosylated IgG having normal PK is demonstrated by the mAb ALD518, which is a humanized antihuman IL-6 IgG1 produced in yeast. In a Phase I clinical trial, the circulating half-life for ALD518 was 20–32 days, consistent with a normal human serum IgG1 half-life.[79] Onartuzumab (*E. coli* produced and thereby aglycosylated), a one arm mAb against the receptor tyrosine kinase MET has been shown to have a 8–12-day half-life in a Phase I clinical trial, shorter than commonly reported glycosylated IgG1. However, the reason for the half-life difference is not clear.[80,81]

The observations of similar PK properties between glycosylated and nonglycosylated IgG suggest that the primary factor determining the PK of an IgG (i.e., FcRn-mediated recycling) is not impaired by glycan removal. Indeed, aglycosylated IgG mAb showed similar FcRn-binding affinity to that of the glycosylated versions.[78,82,83] A mutational analysis of IgG molecules demonstrated that the FcRn-binding site is located at the CH2–CH3 domain overlapping with the protein A-binding site, consistent with the finding that aglycosylated IgG can still bind to protein A and *E. coli* produced IgG exhibits similar PK to that of glycosylated versions produced from mammalian cells.[84]

The Impact of Terminal Mannose and Sialic Acids on the PK of mAb

In general, the glycosylation site of IgG Fc is relatively sequestered and buried in the CH2–CH3 domain, making interaction with glycan-binding receptors sterically unfavored.[15] However, in certain situations, when specific terminal sugars are abundant and possibly exposed, the PK of an antibody could be altered because of glycan binding to receptors present on tissues, usually resulting in rapid removal of antibody from circulation. Glycan receptors that have been attributed to the removal of glycoproteins *in vivo* include the mannose receptor (ManR) and the asialoglycoprotein receptor (ASGPR). The ASGPR and the ManR are carbohydrate-specific, endocytic receptors expressed by hepatic parenchymal (hepatocytes) and nonparenchymal (such as Kupffer) cells and sinusoidal endothelial cells, respectively.[85,86] Glycosylated mAbs with terminal high-mannose glycans have been shown to exhibit fast clearance from the blood.[18,78,87–90] A rabbit antibody with exposed terminal Gal (after removal of sialic acid) has also been shown to be rapidly cleared from the blood and localized in the liver.[91]

A typical CHO-derived recombinant IgG has abundant G0F, low G1F/G2F, negligible sialic acids with some materials having some high-mannose (M5–M9) structures.[18,92–94] Levels of high-mannose glycans on recombinant antibodies can range from 1% to greater than 20%, whereas endogenous human IgG contains only trace levels (<0.1%) of high-mannose glycans.[95] Terminal high-mannose glycans can have a significant impact on IgG PK. Wright and coworkers[88,89] demonstrated that IgG with glycans terminated with Man5 cleared significantly faster than those with more complex glycosylation such as G0F, G1F, and G2F. Kanda et al.[87] showed that the binding affinity of high mannose (more than five mannose residues, typically Man5/8/9) containing IgG to human FcRn was not different from that of hybrid (with both high mannose and complex type) or more complex glycans (with more than two GlcNAc and variable amounts of Gal and sialic acid). However, the PK of these mAbs in mice was significantly different with 14, 8.5, and 4.6 day half-lives for mAbs with complex, hybrid, and high-mannose, respectively. Similar results were obtained with yeast-produced IgG1 mAb with high-terminal mannose content despite having similar FcRn-binding affinities.[78] With more homogenous Man5 and Man8/9 containing IgG mAb, Yu et al.[90] showed similar findings in that mAb with Man5 exhibited much faster clearance than that with complex glycan. The PK of a mAb with Man5 was similar to that with Man8/9 (Fig. 4). Because of abundant expression of ManR and ASGPR in the liver, it is expected that terminal mannose or Gal containing glycoproteins will preferentially demonstrate liver distribution. Indeed, Wright et al.[89] showed that IgG bearing exposed terminal Gal or Man5 tend to distribute to liver and catabolized there, whereas terminal GlcNAc-bearing mAb did not show this tendency.

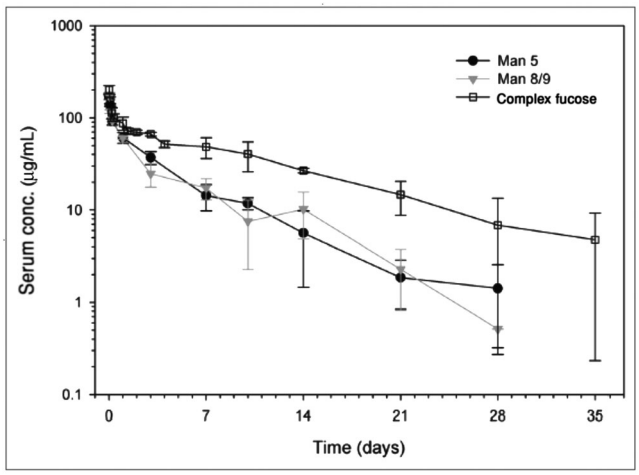

Figure 4. The impacts of high-mannose structure on the PK of a mAb. Nude mice were injected with single i.v. dose of 10 mg/kg mAbs with specific glycoform and serum mAb concentration measured. Man5, mAb with more than 99% Man5; Man8/9, mAb with more than 99% Man8/9; complex Fuc, mAb with complex Fuc. Reproduced from Yu et al.[90] with permission from www.tandfonline.com.

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The aforementioned results came from animal studies with mAbs that were deliberately manufactured with high mannose. Normally, mAbs for clinical use possess relatively low-terminal high-mannose glycan content. Chen et al.[96] reported a higher clearance rate for high-mannose glycoform containing mAbs in a clinical trial but they attributed the change in clearance rate to glycan cleavage *in vivo*, and not to differential clearance of specific types of high mannose glycoform. However, a subsequent study using more refined analytical methods and an extended study length demonstrated that high-mannose glycans containing mAbs were cleared faster.[18] Specifically, therapeutic IgGs containing high-mannose glycans were cleared more rapidly in humans than those with other glycan forms, although the differences in exposure were shown to be relatively minor for three of the four molecules studied. However, depending on the dosing regimen and the relative level of the high-mannose glycan, this can also have significant impact on exposure. Mab4 with up to 17% Man5 showed a 6% reduction in area under the curve (AUC), which could potentially contribute to the inconsistency of efficacy.[18] Thus, high-mannose content of therapeutic mAbs should be considered as an important product quality attribute that may affect PK properties. These studies further suggest that, in spite of generally being regarded as sequestered inside the CH2 domain, high-terminal mannose content appears to be sufficiently accessible to interact with ManRs.

Human serum IgG is typically less than 10% sialylated, whereas recombinant mAb generated from CHO cell lines has negligible sialylation. However, mAb produced in mouse hybridoma cells can be up to 50% sialylated.[38] Naso et al.[97] created high and low sialic acid bearing mAbs by manipulation of sialidase in the host cells and showed no difference in the PK between low- and high-sialylated mAb. Scallon et al.[98] generated mAbs from mouse hybridoma cell lines with terminal sialylation varying from 0% to 90% and demonstrated that sialylation interfered with binding to FcγRIIIa, leading to reduced ADCC. However, PK were not evaluated for these mAbs.

In addition to Fc glycosylation, about 15%–20% of human serum IgG molecules bear N-linked oligosaccharides in the variable region.[99] However, the vast majority of recombinant therapeutic mAbs are only glycosylated at the Fc region with the exception of cetuximab that also has N-linked glycosylation at Asn[88] of the VH region.[9] Huang et al.[100] investigated an anti-Aβ mAb that had additional N-glycosylation in the Fab domain with terminal sialic acid and showed that fragment, variable (Fv) glycosylation did not appear to affect the PK of the mAb they studied. However, depending on the position of the glycosylation, Fv carbohydrates can have an impact on the clearance of IgG antibodies. Coloma et al.[101] evaluated some anti-dextran antibody variable region glycovariants and showed that in comparison to other Fv glycovariants, only a glycovariant with two specific Fv position (VL28/VH58) glycosylation exhibited significantly faster clearance, which correlated with high liver uptake and the mechanism was attributed to ASGPR mediated clearance. Presumably, the specific site of glycosylation in the Fv region has caused conformational changes that may have facilitated the binding of the mAb to the ASGPR in the liver.

Glycosylation Impact on the PK of Fc-Fusion Proteins

The primary purpose of linking a therapeutic protein to an Fc fragment is to prolong the circulating half-life of the partner molecule that would otherwise be rapidly cleared from the circulation. However, the PK behaviors of Fc-fusion proteins depend not only on Fc, but also on the fusion partner and its associated glycosylations. IgG1Fc is the most commonly used Fc for Fc-fusions. The partner molecules range from large proteins to small peptides. Most of the partner proteins are heavily glycosylated, whereas some Fc partner peptides may not be glycosylated at all.[102] Many of the Fc-fusion partners comprise the extracellular domains of cellular receptors for blocking cognate receptor–ligand interactions for therapeutic activities. Table 2 lists approved Fc-fusion proteins.

It is well known that the circulating half-life of Fc-fusions is significantly shorter than that of whole IgG molecules. The reasons may be at least threefold: (1) the affinity of Fc to FcRn is significantly reduced because of fusion; (2) the liability of glycans in the fusion partner because of glycan receptor-mediated disposition; and (3) receptor-mediated clearance by partner molecule. To investigate the mechanism for the differential clearance *in vivo* between intact antibody and Fc-fusion molecules, Suzuki et al.[103] compared binding affinity at pH 6.0 to FcRn of several Fc-fusion products to those of IgG mAb products. They demonstrated that the binding affinity of Fc-fusion proteins to FcRn was significantly reduced in comparison to intact IgG. In one example, the authors demonstrated that the binding to FcRn by the Fc domain of etanercept (TNFRII-Fc-fusion) could be increased through the removal of the tumor necrosis factor α receptor II (TNFRII) ectodomain by papain digestion. Thus, the authors suggested that the Fc-fusion partner, the TNFRII ectodomain, influenced the structural environment of the FcRn-binding region, thus leading to a reduced association between the Fc domain and the FcRn. However, the FcRn binding is probably only one part of the equation determining the PK properties of Fc-fusion molecules. Glycosylation patterns may play a more important role in determining the *in vivo* clearance. For example, in the investigation of humanized yeast-produced TNFαRII-Fc-fusion molecules, it was demonstrated that it was the extent of sialylation on the TNFRII, not the FcRn-binding affinity, that determined the clearance. The exposure was positively correlated to the quantity of the sialylation on the receptor molecule with higher sialic acid content resulting in higher exposure.[17] Figure 5 presents

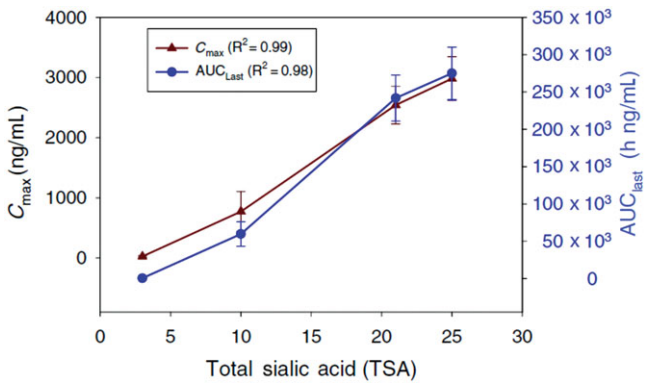

Figure 5. The impacts of sialic acid NANA on the PK of etanercept. Rats were injected subcutaneously with etanercept glycovariants with different amount of total sialic acid NANA. Serum etanercept concentrations were measured and AUC_{last} and C_{max} determined. Reproduced from Liu et al.[17] with permission from Springer Science+Business Media.

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the relationship between AUC/C_{max} and total sialic acid content.

There are several other Fc-fusion molecules that also rely on sialylation in reducing the *in vivo* clearance. BR3–Fc-fusion has multiple O-linked glycosylation sites, and it has been shown that changes of the sialic acid content through the manufacturing process have a direct relationship with the PK properties.[11] BR3–Fc molecules with high sialic acid content exhibited much higher systemic exposure following i.v. administration, when compared with those molecules possessing lower sialylation levels or when desialylated. The rapid clearance of low sialic acid containing or desialylated BR3–Fc was attributed to the binding of the exposed Gal to the ASGPRs and was subsequently degraded in the liver in nonparenchymal cells (Kupffer cells).[11] Interleukin 23 receptor Fc-fusion protein (IL-23R-Fc) produced in human embryonic kidney (HEK) cells and CHO cells have differential PK properties with HEK cell product having 50% of the half-life of CHO product (terminal half-life 44 vs. 90 h). Analytical analysis indicated that the version produced in HEK cells had a significantly lower content of total sialic acids.[104]

Human cytotoxic T lymphocyte-associated protein 4 (CTLA4)-Ig is an IgG1 Fc-fusion with extracellular domain of CTLA4. Flesher et al.[105] showed that CTLA4-Ig with a higher total sialic acid content exhibited significantly slower clearance than those with lower sialic acid content. After removal of sialic acid from CTLA4-Ig, rapid clearance from circulation was observed.[105] Interestingly, in the same report, it was shown that the type of sialic acids also matters. Unlike the products produced in CHO cells, which has predominately terminal NANA, the products produced in NS0 cells have NGNA. When tested *in vivo*, murine CTLA4-Ig produced in NS0 cells exhibited much faster clearance than that produced in CHO cells.[105] Similar findings were observed with lymphocyte function-associated molecule 3 (LFA3)TIP, a fusion of the LFA 3, and the Fc-domain of IgG1. When LFA3TIP produced in murine NS0 myeloma cell lines was compared with the same molecule produced in CHO, a significant reduction in half-life of the molecule was observed for NS0 produced material because of the lack of NANA, whereas CHO-derived materials had high levels of NANA.[106] Plant cells are not naturally capable of making sialic acid NANA; thus, CTLA4-Ig produced in rice has significantly faster clearance than that derived from CHO cells.[107]

Other terminal monosaccharide such as GlcNAc can also contribute to the PK properties of Fc-fusion molecules. Jones and coworkers[108,109] reported that the PK properties of TNFαRI-Fc (lenercept) appeared to be negatively correlated to the content of terminal GlcNAc. However, their studies did not exclude the critical role of sialic acids because all the batches they characterized had similar levels of sialic acid.

The term sialic acid is commonly used to describe both NANA and NGNA, it is important to recognize that only NANA, and not NGNA, has such a critical role in the PK of Fc-fusion molecules. The contribution of NANA to the PK of Fc-fusion proteins appears to be applicable to either O- or N-linked glycans as Fc-fusion partners of lenercept, CTLA4-Ig, LFA3TIP, and IL23R-Fc are N-glycosylated, whereas those of etanercept and BR3-Fc are O-glycosylated. The underlying mechanism for the protective role of NANA is to cap the Gal to prevent the associated proteins from being recognized by ASGPR in the liver and removed from circulation.[110]

GLYCOSYLATION IMPACT ON PD OF mAb

Glycosylation Is Required for Fc Receptor-Mediated Effector Functions of IgG Antibodies

The effects of glycosylation on PD of antibodies were reported as early as in 1973 when Williams et al.[111] used glycosidase to remove glycans and tested the effect on complement fixing and other Fcγ receptor-associated activities. The results showed that some antibodies were completely dependent on the presence of glycosylations for activity. More definitive results of removing glycans on antibody function came from Koide et al.,[112] when they showed that deglycosylated rabbit antibodies lost the ability to activate ADCC and the binding to complement. A more comprehensive study was conducted by Nose and Wigzell[76] with tunicamycin-aglycosylated mouse IgG2b molecules, demonstrating the absolute requirement of glycosylation on Fc receptor binding, complement activation, and ADCC activities with little effect, if any, on antigen binding and protein A binding. With genetically engineered mouse–human chimeric IgG1 and IgG3 antibodies that have the Asn^{297} site mutated to prevent glycosylation, Tao and Morrison[15] showed that aglycosylated IgG1 and IgG3 lost the ability to bind FcγRI receptors and to activate complement. Many subsequent studies using humanized IgG and fully human antibodies all confirmed the criticality of glycosylation on Fc receptor-mediated effector functions (see below).

Influence of Glycoforms on Fc Effector Functions

The composition and patterns or glycoforms could have positive or negative impact on the Fc effector functions. The most striking impact of glycans on Fc effector functions is the one mediated by Fuc, which is added on to the core structure in the late stages of glycan biosynthesis in the Golgi apparatus (see section *IgG Glycan Structures and Biosynthesis*). Core fucosylation inhibiting IgG binding to FcγRIIIa and decreasing ADCC activity was suggested in the study of Rothman et al. in 1989.[113] With the Lec 13 CHO cell line that is deficient in adding Fuc to its core glycosylation, Shields et al.[114] demonstrated that afucosylated IgG mAbs exhibited a 50-fold increase in binding to the Fcγ RIIIa receptor, and that ADCC activity was enhanced greatly as well. It was also shown that the binding to FcγRI, C1q, or FcRn were not altered. Many subsequent studies confirmed this finding and the methods to produce afucosylated IgG were extensively investigated.[42,43,115–122] The first patent related to ADCC enhancement with afucosylated IgG was published in 2000.[123] Using marketed mAbs such as rituximab and trastuzumab, it has been definitively demonstrated that defucosylation increases ADCC activities by at least two orders of magnitude in humans *in vitro* and *ex vivo*.[43,114,122] It was concluded that the Fuc residue attached to the innermost GlcNAc of the biantennary complex-type oligosaccharides plays the most critical role in the ADCC of therapeutic antibodies. The ADCC activity of afucosylated IgG appears to be already maximized because any higher binding affinity generated by amino acid mutation could not generate higher ADCC activity.[122,124] For instance, triple amino acid-substituted rituximab mutant (S239D/S298A/I332E) with approximately 10-fold higher affinity for FcγRIIIa than that of nonfucosylated rituximab, was shown to exhibit identical ADCC activity to that of nonfucosylated rituximab in a human *ex vivo* model

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as demonstrated by monitoring rituximab-induced B cell depletion from human whole-blood samples.[122] The significantly enhanced ADCC activity *in vivo* was attributed to the enhanced Fcγ RIIIa-binding affinity by the afucosylated IgG Fc; without significantly higher affinity, small amount of therapeutic mAb are usually competed out by the large pool of endogenous circulating IgG for binding to Fcγ receptors, resulting in no therapeutic effects.[122] Therefore, a therapeutic mAb with desired ADCC activity for its therapeutic effects requires significantly higher affinity to FcγRIIIa than endogenous IgG.

Including a bisecting GlcNAc glycan structure at Asn[125,297] has been shown to enhance the binding affinity to FcγRIIIa, resulting in 10–20-fold higher ADCC activities. However, *in vivo* addition of bisecting GlcNAc and core Fuc are biosynthetically linked as core Fuc addition is inhibited by prior addition of bisecting GlcNAc.[126] Therefore, *in vivo* addition of bisecting GlcNAc always results in loss of core fucosylation. It has been debated whether the demonstration that bisecting GlcNAc increases ADCC activity may not be solely attributed to bisecting GlcNAc alone.[125] Using the rat hybridoma cell line YB2/0, which contains low fucosyltransferase activity and a CHO cell line that fully fucosylate mAb, Shinkawa et al.[40] showed that not the presence of bisecting GlcNAc or Gal but the absence of Fuc was critical for mAb's ADCC activity; addition of bisecting GlcNAc exhibited only several fold improvement of ADCC activity, whereas lack of Fuc increased the ADCC activity about 50-fold.

To elucidate the role of bisecting GlcNAc in ADCC without the complex *in vivo* situation, Hodoniczky et al.[127] used an *in vitro* remodeling method to add bisecting GlcNAc without removal of core Fuc and demonstrated that rituximab with bisecting GlcNAc and intact core fucosylation improved ADCC activity by approximately 10-fold, indicating that a bisecting GlcNAc can specifically enhance ADCC activity independent of any other N-glycan modification. Moreover, they confirmed the results with trastuzumab in that trastuzumab-bearing N-glycans maximally modified with a bisecting GlcNAc showed elevation in ADCC (10-fold), which was comparable to that observed for similarly remodeled rituximab.

Terminal Gal does not seem to play an important role in the ADCC activity of an IgG mAb, although small changes could be detected after degalactosylation.[127–130] However, Gal has been shown to play an important role in CDC activity. With rituximab, CDC increases with increase in Gal content in the heavy chain and the affinity to C1q increased twofold from G0 to G2.[130] With alemtuzumab, the removal of Gal reduced CDC activity.[128] Ferrara et al.[131] also showed that one IgG1 mAb without core Fuc exhibited high ADCC activity but reduced CDC activity, whereas another mAb with bisecting GlcNAc (also lack of Fuc) had increased ADCC activity but not changes in CDC activity compared with a fucosylated control.

High-mannose contents (Man5/8/9) in an IgG mAb results in higher binding affinity to FcγRIIIa and hence higher ADCC activity.[87,90,132,133] The higher ADCC activity, however, may be because of the absence of Fuc because high-mannose glycans do not have core fucosylation.[133] Core fucosylation requires the GlcNAc added by GnT-1; therefore, only hybrid and complex glycans can become core fucosylated. Yu et al.[90] showed that high-mannose glycans increased the affinity to FcγRIIIa, resulting in enhanced ADCC activity; high mannose also reduced binding to C1q, leading to lower CDC activity.[90,132–134]

Scallon et al.[98] generated mAbs from mouse hybridoma cell lines with various contents of terminal sialylation (NGNA) from 0% to 90%, and demonstrated that sialylation interfered with binding to FcγRIIIa, leading to reduced ADCC. Pairs of mAbs that differ in the amount of sialic acid (NGNA) in their Fc glycans were examined for the relationship between the amount of NGNA and ADCC activity and the results showed that, for each of the three Ab pairs examined, higher levels of sialylation were associated with reduced activity in an ADCC assay.[98] Although the type of sialic acid was not specified in the paper, it is assumed that it was NGNA because the production host was murine myeloma cell lines.[37]

Sialylation of IgG has also been shown to be important for the anti-inflammatory effect of intravenous immunoglobulin G (IVIG) for the treatment of autoimmune and inflammatory diseases.[135] Although more than one mechanism may be involved in the anti-inflammatory effect of IVIG, facilitated interactions to C-type lectin-like receptor-specific intracellular adhesion molecule-grabbing nonintegrin R1 (SIGN-RI) (DC-SIGN in humans) by sialylated IgG (~10% sialylated in the IVIG preparations) leading to an increased FcγRIIb expression and the suppression of inflammatory response, has been demonstrated.[136–140] The anti-inflammatory effect of IVIG was recapitulated with recombinant IgG1 with 2, 6 sialylation,[141] which may pave the way for IVIG replacement therapy with recombinant sialylated IgG Fc for the treatment of autoimmune and inflammatory diseases. The unique glycosylation patterns of natural circulating IgGs have been observed in some physiological and pathophysiological conditions. The levels of sialylation and galactosylation of IgG have been shown to increase in pregnancy-related remission of rheumatoid arthritis and decrease in aging populations and in patients with autoimmune diseases, infectious diseases, and tumors.[142–147]

Although the glycan impact on the effector function of an IgG molecule is associated with Fc glycans, glycosylation in the variable region can influence antibody function as well. It has been shown that depending on the sites where the glycosylation occurs, glycosylation in the Fv region can either significantly enhance or reduce the antigen-binding activities of an antibody.[148,149] Jacquemin et al.[150] isolated an anti-FVIII mAb from a hemophilia A patient who had high-antibody titer against FVIII and the mAb showed a strong neutralizing activity. This mAb had N-linked glycosylation in the Fv region and deglycosylation led to 50% reduction of neutralizing activity despite similar binding affinity to FVIII.[150] Asymmetric mAb glycosylation (single arm glycosylation) has been reported for an IgG1 with a single glycosylation in the Fab region, resulting in the loss of its divalent binding ability to the antigen.[151] Differential glycosylation patterns between Fab and Fc have been found in the serum IgG of pregnant women in that the levels of galactosylation, sialylation, incidence of bisecting GlcNAc, and the presence of a high-mannose structure were all substantially higher in the Fab than in the Fc, whereas Fc had higher levels of fucosylation.[152]

Afucosylated Antibodies as Next-Generation Therapeutics for Cancer Therapy

For therapeutic antibodies requiring killing of target cells, ADCC, CDC, and direct cell apoptosis are critical for

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efficacies. ADCC appears to be particularly important for cancer cell targeting mAbs such as rituximab and trastuzumab. Human IgG1 binds more strongly to homozygous FcγRIIIa-158V NK cells than to homozygous FcγRIIIa-158F or heterozygous NK cells.[153] The binding affinity appears to correlate with clinical efficacies in that patients with FcγRIIIa-158V alleles showed higher response rates than those with FcγRIIIa-158F alleles.[154–158] This correlation has also been demonstrated in RA patients treated with rituximab in that the response rates by FcγRIIIa 158V/V allotype patients were higher than F/F allotype-bearing patients.[159] Breast cancer patients who responded to trastuzumab with complete or partial remission were found to have a higher capability to mediate *in vitro* ADCC with trastuzumab than nonresponders; and the FcγRIIIa-158 V/V genotype was significantly correlated with objective response rate and progression-free survival (PFS).[160] FcγIIIa-158V/V genotypes had longer PFS than 158F carriers in the treatment of colorectal cancer by cetuximab.[161] Because of these observations, there have been intensive investigations to produce mAbs that have enhanced ADCC activities. Removal of Fuc from IgGs is by far the most powerful method to enhance ADCC activity. In the last decade, the biopharmaceutical industry has vigorously pursued the generation of afucosylated mAb therapeutics. Genetic elimination of fucosyltransferase 8 (FUT8) in CHO cells has been a successful approach for the prevention of core fucosylation of IgG.[115] Alternative methods such as the overexpression of β (1,4)-N-acetylglucosaminyltransferase III (GnTIII) as a means to eliminate the substrate for fucosyltransferase and modification of enzymes involved in the N-linked glycan biosynthetic pathway have also been developed with significant success.[133,162,163] Expression of mAb in some host cells that are naturally lacking Fuc such as yeast has also been explored for the production of afucosylated IgGs.[43] Many biopharmaceutical companies have developed glycoengineering platforms and numerous glycoengineered biobetter mAbs are in various stages of clinical development.[164] Mogamulizumab was the first glycoengineered mAb obtaining marketing approval from the Pharmaceuticals and Medical devices agency (PMDA) of Japan in 2012. Mogamulizumab is a humanized anti-CCR4 (C-C chemokine receptor type 4) mAb approved for the treatment of patients with relapse or refractory CCR4-positive T cell leukemia or lymphoma. Manufactured in FUT8 knock out CHO cells (POTELLIGENT® Technology) without core Fuc, mogamulizumab possesses significantly enhanced ADCC activity and excellent clinical efficacy.[165]

Although mogamulizumab was the first approved glycoengineered mAb, no head to head comparison with a nonglycoengineered version was conducted in the clinical trials. The demonstration of the superiority of a glycoengineered mAb over that of the nonglycoengineered version came from the US approval of Gazyva® (obinutuzumab, GA101).[20] Gazyva® is the third-generation type II anti-CD20 humanized mAb that was approved by the FDA in November, 2013 for the treatment of patients with previously untreated CLL. Gazyva® is manufactured with the GlycoMab™ platform utilizing antibody-producing cells overexpressing heterologous GnTIII that blocks the further glycan biosynthesis such as core Fuc addition and conversion of a hybrid structure to a complex structure.[164] In addition, modification of elbow hinge sequences within the antibody variable framework regions results in a strong apoptosis-inducing activity of obinutuzumab upon CD20 binding on target cells. The bisecting and afucosylated mAb showed about 50-fold higher affinity to human FcγRIII receptors compared with a standard, nonglycoengineered antibody. Increased FcγRIIIa binding led to a 10–100-fold increase in ADCC against CD20-expressing NHL cell lines.[19,166] The registration trials compared obinutuzumab in combination with chlorambucil with rituximab in combination of chlorambucil.[20,167] The overall response rate was higher with obinutuzumab/chlorambucil versus rituximab/chlorambucil: 78% versus 65%. Obinutuzumab/chlorambucil was also superior in eradicating detectable disease with the rate of minimal residual disease with negative status in bone marrow being 19.5% versus 2.6% and in blood being 37.3% versus 3.3% for obinutuzumab/chlorambucil and rituximab/chlorambucil, respectively. For the primary endpoint, obinutuzumab/chlorambucil led to a 61% improvement in the likelihood of being progression-free during the study follow-up so far, compared with rituximab/chlorambucil. Median PFS was 26.7 versus 15.2 months in these two groups, respectively, and 11.1 months for the chlorambucil-alone control arm. Table 4 summarizes the key trial results.

Table 4. Gazyva (Obinutuzumab, GA101) Registration Trial Key Results

	Obinutuzumab + Chloramubucil	Rituximab + Chlorambucil	*p* Value
ORR (%)	78	65	<0.0001
Median PFS (month)	26.7	15.2	<0.0001
Minimal Residual Disease			
Bone marrow (%)	19.5	2.6	<0.0001
Blood (%)	37.3	3.3	<0.0001

Based on Goede et al.[20]
PFS, progress free survival; ORR, overall response rate.

GLYCOSYLATION IMPACT ON IMMUNOGENICITY OF THERAPEUTIC mAb

Therapeutic proteins can be immunogenic and may induce an ADA response.[168] The immunogenicity of therapeutic mAbs can cause hypersensitivity responses including anaphylaxis, infusion reactions, and accelerated clearance of drug, resulting in decreased safety and efficacy. Many factors can influence the immunogenicity of therapeutic mAb. Immunogenicity could be caused by altered protein structures such as amino acid difference/modifications, aggregations, neoepitopes formation in fusion protein junctions, formulations, and glycan compositions.[169]

High-mannose-type N-glycans are commonly found in glycoproteins derived from yeasts, insect cells, and plants and could be highly immunogenic in humans.[44] It has been shown that antigen-bearing mannan (polymer of mannose) was significantly more immunogenic than that without mannan.[170] Although the majority of therapeutic mAb contain very low level of high-mannose structure, some may contain high-mannose glycans.[18] Therefore, care must be taken to control the mannose content in the therapeutic protein to avoid potential immunogenicity.

Therapeutic mAbs produced in mouse myeloma cells may contain α-Gal epitope (Galα1–3Galβ1–4GlcNAc-R) present

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in the glycosylation.[39,171] The α-Gal epitope is abundantly expressed on glycoconjugates of nonprimate mammals, prosimians, and New World monkeys but not on glycoconjugates of Old World monkeys, apes, and humans. As a result, nonexpressers produce large amounts of natural anti-Gal antibody that specifically binds the α-epitope. It is estimated that anti-Gal antibody is the most abundant natural antibody in humans, apes and Old World monkeys, constituting approximately 1% of circulating immunoglobulins.[171] It is a general concern that mAbs bearing α-Gal epitope would be immunogenic in humans. Indeed, Erbitux® (cetuximab), manufactured in murine myeloma cells (SP2/0), contains α-Gal epitope and is immunogenic in humans. The marketed version of cetuximab has been reported to contain 21 different glycoforms with around 30% capped by at least one α-1,3-Gal residue, 12% capped by a NGNA residue and traces of oligomannose.[172] It has been reported that cetuximab induced anaphylaxis in some of the treated patients and pre-existing IgEs specific for this α-Gal epitope were detected in these patients.[173,174] Interestingly, α-Gal epitope was also reported to be the potential cause of red meat allergies in humans in certain geographical regions where tick bites were shown to induce IgE antibody response to α-Gal, resulting in delayed anaphylaxis.[175–177]

Recombinant glycoproteins produced in nonhuman mammalian cell lines such as murine myeloma cell lines are often modified with the nonhuman sialic acid NGNA.[37,172] The presence of NGNA has generally been ignored in drug development because healthy individuals were not thought to react to NGNA. However, recent findings indicate that all humans have NGNA-specific antibodies, sometimes at high levels.[178–180] Cetuximab, an anti-EGFR (epidermal growth factor receptor) mAb, is manufactured in murine myeloma cells known to contain NGNA, whereas panitumumab (also an anti-EGFR mAb) is produced in CHO cells with negligible NGNA. Anti-NGNA antibodies from healthy humans interact with cetuximab in a NGNA-specific manner and generate immune complexes *in vitro*. In contrast, these antibodies did not show reactivity toward panitumumab.[181,182] Mice with a human-like defect in NGNA synthesis generate antibodies to NGNA after injection with cetuximab, and circulating anti-NGNA antibodies can promote drug clearance.[181,182] It has been suggested that red meat containing NGNA could be incorporated into human tissues and induce chronic inflammation, possibly contributing to the high frequency of diet-related carcinomas and other diseases in humans.[178] Therefore, mAbs with glycans that contain nonhuman sialic acid NGNA may have potential to cause immunogenicity in human subjects.

GLYCOSYLATION AND REGULATORY CONSIDERATIONS OF BIOSIMILAR mAb AND Fc-FUSION PROTEIN DEVELOPMENT

As discussed in the above sections, the glycosylation patterns or glycoforms of therapeutic mAbs or Fc-fusion proteins can be quite heterogeneous depending on the expression system and the bioprocess used. Even expressed in the same system, the glycoforms of an antibody can be different for different clones. To reduce the price burden of highly efficacious biologics drugs, some countries have enacted biosimilar drug development laws, and health agencies including FDA and EMEA have provided biosimilar drug development guidelines. The FDA defined biosimilar drug as "the biological product is highly similar to the reference product notwithstanding minor differences in clinically inactive components and that there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product."[21–24] EMEA has similar requirements for the development of biosimilars.[25,26]

The FDA emphasized three key concepts in the biosimilar development: exposure and response assessment, evaluation of residual uncertainty, and assumptions about analytical quality and similarity.[24] Glycosylation was cited as an example in FDA similarity definitions:

> "As an example, glycosylation plays an important role in the PK of certain protein products. Manufacturing process conditions may impact glycosylation. Comparative PK and PD studies of the proposed biosimilar product and the reference product help resolve that some differences in glycosylation identified in the analytical studies would be within an acceptable range to consider the proposed biosimilar product to be highly similar to the reference product."[24]

The quality attributes of glycosylation patterns can determine whether the biosimilar candidate is "highly similar" so that the preclinical animal studies or clinical studies can be "targeted and selective" or is "highly similar with fingerprint-like similarity" so that the animal studies or clinical studies can be "more targeted and selective."[24]

Manufacturing processes change quite often because of process improvement, scale elevation, and site changes, even after product approval.[183] The resulting changes in quality attributes need to be carefully controlled and the changes should not affect product's safety and efficacy. Microheterogeneity of glycosylation is one of the critical quality attributes that can determine whether the product can maintain its original safety and efficacy after changes. By analyzing the quality profiles of the glycosylated recombinant therapeutic proteins Aranesp® (darbepoetin alfa), Rituxan®/Mabthera® (rituximab) and Enbrel® (etanercept) sourced from the market between 2007 and 2010, Schiestl et al.[184] provide some excellent examples of acceptable variations for products that have remained on the market with unchanged product labels. It was shown that commercial rituximab batches exhibited a shift in terms of unfucosylated structure from 0.3% to 0.6% to postchange 0.9% to 1.8% (a threefold change), which correlated to the changes of ADCC activities from 70% to 115% (relative to reference) to postchange 108% to 129%. The amount of variants of Enbrel® containing the N-glycan G2F decreased from approximately 50% in the prechange to approximately 30% in the postchange material. As no changes were made to the labels, the observed variations of glycosylation were predicted not to result in an altered clinical profile. Product quality-attribute changes after market approval is analogous to the quality attribute variations of biosimilars to the original products. During biosimilar drug development, it will be beneficial to determine the range of quality attributes of the innovator materials over time and use the range as a reference to conduct similarity exercises between biosimilars and the original products.

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Table 5. Summary of Key Impacts of Glycosylation on the PK and PD of mAb and Fc-Fusion Proteins

Glycan	Impacts
Mannose	• Increases the clearance of mAb[18,78,87–90]
	• Enhances FcγRIIIa binding/ADCC of mAb[87,90,132,133]
	• Reduces C1q binding/CDC of mAb[90,132–134]
Fucose	• Interferes with binding to FcγRIIIa
	• Defucosylation enhances FcγRIIIa binding/ADCC activity[42,43,113–122]
Galactose	• Exposed galactose may increase the clearance of mAb[89,91]
	• Enhances CDC of mAb[128,129]
GlcNAc	• Bisecting GlcNAc enhance FcγRIIIa binding/ADCC[127]
	• Increases the clearance of Fc-fusion proteins[108,109]
Sialic acid NANA	• Critical for reducing the clearance of Fc-fusion proteins[11,17,104–107]
	• Anti-inflammatory activity[135–141]
Sialic acid NGNA	• Interferes with FcγRIIIa binding and reduce ADCC activity of mAb[98]
	• May be immunogenic in humans[180–182]
Galα1–3Galβ1–4GlcNAc-R	• Immunogenic in humans and may induce anaphylaxes[173,174]

SUMMARY AND CONCLUSIONS

Therapeutic antibodies and Fc-fusion molecules have shaped the clinical practice in almost every area of medicine. The high efficacy and safety profiles offered by this class of drugs have propelled tremendous growth of this type of medicine. Antibodies are naturally glycosylated and this glycosylation plays a critical role in antibody structure and functions. Most therapeutic monoclonal antibodies are of IgG class that has a glycosylation site in the Fc region at amino acid position 297 and may also have glycosylation in the Fab region. For Fc-fusion proteins, glycosylation also occurs in the fusion partners. Depending on the expression host, the glycosylation composition and patterns or glycoforms in a mAb or Fc-fusions can be significantly different, which can have significant impacts on the PK and or PD of monoclonal antibodies, resulting in potentially altered efficacy and safety profiles (Table 5). MAb produced in normal CHO cells are fucosylated, whereas mAbs produced in yeast or in rat YB2/0 cells are afucosylated or with low fucosylation, resulting in much more potent ADCC activity. Glycoengineering in CHO expression system can produce nonfucosylated mAbs that are highly potent in ADCC. Although the primary determinant of IgG's long-circulating half-life is FcRn, which rescues IgG molecules via pH-dependent recycling, other factors including terminal glycans, charges, and aggregations can also have impacts. Glycosylation itself is not required for maintaining IgG's long half-life. However, terminal mannose or sialic acids can have significant impact on PK of mAb or Fc-fusion proteins. Mannose, especially Man5/8/9 content in the mAb, can have significant impact on the PK of the molecule, potentially reducing the exposure, leading to lower efficacy. The sialylation level has significant impact on PK of Fc-fusion molecules in that lower sialylation can result in lower exposure. Glycosylation is one of the critical quality attributes that can impact the development of biosimilar mAbs and Fc-fusion molecules. As glycosylation patterns can change with different expression system, culture conditions, processes, and scales of manufacture, close control of glycosylation of biosimilar product candidates is required to ensure high similarity in safety and efficacy to the innovator molecules.

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