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REVIEW
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Role of Fc Core Fucosylation in the Effector Function of IgG1 Antibodies

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The presence of fucose on IgG1 Asn-297 N-linked glycan is the modification of the human IgG1 Fc structure with the most significant impact on FcγRIII affinity. It also significantly enhances the efficacy of antibody dependent cellular cytotoxicity (ADCC) by natural killer (NK) cells *in vitro*, induced by IgG1 therapeutic monoclonal antibodies (mAbs). The effect of afucosylation on ADCC or antibody dependent phagocytosis (ADCP) mediated by macrophages or polymorphonuclear neutrophils (PMN) is less clear. Evidence for enhanced efficacy of afucosylated therapeutic mAbs *in vivo* has also been reported. This has led to the development of several therapeutic antibodies with low Fc core fucose to treat cancer and inflammatory diseases, seven of which have already been approved for clinical use. More recently, the regulation of IgG Fc core fucosylation has been shown to take place naturally during the B-cell immune response: A decrease in α-1,6 fucose has been observed in polyclonal, antigen-specific IgG1 antibodies which are generated during alloimmunization of pregnant women by fetal erythrocyte or platelet antigens and following infection by some enveloped viruses and parasites. Low IgG1 Fc core fucose on antigen-specific polyclonal IgG1 has been linked to disease severity in several cases, such as SARS-CoV 2 and Dengue virus infection and during alloimmunization, highlighting the *in vivo* significance of this phenomenon. This review aims to summarize the current knowledge about human IgG1 Fc core fucosylation and its regulation and function *in vivo*, in the context of both therapeutic antibodies and the natural immune response. The parallels in these two areas are informative about the mechanisms and *in vivo* effects of Fc core fucosylation, and may allow to further exploit the desired properties of this modification in different clinical contexts.

Keywords: therapeutic antibodies, IgG, N-glycan, fucosylation, ADCC, NK cells, virus, humoral response

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INTRODUCTION

IgGs are among the most abundant proteins in the circulation (700-1600 mg/dl in healthy adults), and specific IgGs are induced in response to infection, endogenous or allogeneic challenges, or by vaccination. Different IgG subclasses are found in man, which are very similar structurally but have distinct functions due to their differential binding to FcγRs, complement components as well as other

proteins. About 60% of plasma IgG is IgG1, 32% IgG2 and 4% each IgG3 and IgG4 in humans (1). IgGs are glycoproteins and their glycosylation pattern can change during time, due to age, diseases or environmental factors (2, 3).

Therapeutic monoclonal antibodies (mAbs) have emerged as an important therapeutic option in cancer since the approval in 1997 of the anti-CD20 antibody rituximab for the treatment of B-non Hodgkin's lymphoma (B-NHL). Since then, antibodies directed against different antigens expressed by cancer, immune cells or infectious agents have been developed to treat a variety of diseases. Indeed, so far, over 130 antibodies have been approved by the US and EU Drug Agencies, with 45% for oncological disorders, 27% for immune- or inflammation-related conditions and the rest for infectious or other diseases (4).

Most unconjugated therapeutic mAbs are IgG1 or in some cases IgG4 or IgG2. This is because the human IgG1 Fc moiety interacts efficiently with activating FcγRs (FcγRI, IIA, IIC, IIIA and IIIB), expressed on the surface of immune cells (1, 5). This interaction leads to antibody-dependent cellular cytotoxicity (ADCC) by NK cells (mostly *via* FcγRIIIA, CD16A) (6, 7), antibody dependent phagocytosis (ADCP) by macrophages (mostly through FcγRI, CD64 and to some extent FcγRIIA, CD32A) (8–12) and ADCC/ADCP by polymorphonuclear neutrophils (PMN)(mostly *via* FcγRIIA, CD32A) (13–15). IgG1 also interacts with FcγRIIIB (CD16B), a GPI-linked molecules lacking activating domain, highly expressed by PMN and involved in PMN mediated ADCC and ADCP, but whose role may be either activating or inhibiting, perhaps depending on stimulus (13–16). Immune cell activation *via* FcγRs also induces the release of cytokines and chemokines that may cooperate in eliminating the target cells but also induce unwanted side-effects (17). Finally the Fc region of human IgG1 can bind to the first component of the complement cascade C1q and activate the classical pathway of complement which may lead to cell lysis and death through complement dependent cytotoxicity (CDC), as well as phagocytosis by macrophages and PMN through complement receptors on these cells (18). Therefore, many therapeutic antibodies against cancer cells or other targets are of the IgG1 isotype to allow activation of a panoply of immune-mediated mechanisms, many of which rely on FcγRs.

When the activation of the immune system is not desired, for example when a therapeutic antibody is required only to neutralize the antigen, such as a growth factor or checkpoint inhibitor, then the human IgG4 or IgG2 subclasses are often chosen, because they do not interact efficiently with FcγRs or with C1q. The more recent human IgG4 formats include a mutation in Fc (S228A) to avoid Fab arm exchange, a natural phenomenon that leads to IgG4 instability (19).

Over the last 10-15 years, various modifications of antibody structures have been introduced to increase the efficacy of therapeutic mAbs *in vitro* and *in vivo*: these include extensively modified Abs with additional effector functions, such as bispecific antibodies (bsAbs), antibody-drug conjugates (ADCs) and fusion proteins carrying for example cytokines (17, 20). Less dramatic modifications of therapeutic mAbs include the introduction of point mutations in the Fc domain, as well as modification of Fc N-linked glycans that modulate IgG binding to FcγRs and therefore enhance or abolish Fc mediated immune activation (ADCC, ADCP and/or CDC) (17, 20).

In this paper, we will summarize the knowledge gained about the role of IgG1 N-glycan core fucosylation in the *in vitro* and *in vivo* functions of IgG1 antibodies. Ig isotypes or subclasses other than IgG1 bear N-glycans, but less is known about the role of core Fc fucosylation in their case and these will not be further discussed here. Interestingly, the studies on the role of Fc core fucose in therapeutic IgG1 mAbs has facilitated the detection and understanding of the significance of this modification, observed during the polyclonal IgG1 response to some infectious agents, alloimmunization and in some autoimmune conditions. The knowledge on these aspects will therefore also be summarized and discussed.

THE IGG N-LINKED GLYCANS

Human IgGs are glycosylated proteins with a complex and variable glycosylation pattern. An important and extensively studied N-glycosylation site is present at conserved Asparagine 297 (Asn 297) in the CH2 domain, that interacts with FcγRs. 20-30% of IgGs also bear N-glycans on Fab arms (21). Although there are reports of functional effects of different Fab N-glycosylation patterns in some antibodies (22), these are likely to be mostly antibody specific (23). Detailed structural studies of several commercial therapeutic mAbs has revealed that although Fab interacts with FcγRIIIA and stabilizes the Fc-FcγRIIIA binding, Fab fucosylation has a limited effect on the affinity of IgG for FcγRIIIA (23, 24). The modulation of Fab fucosylation will therefore not be further discussed here.

The IgG Asn 297 N-glycans show a high degree of microheterogeneity, and they can be grouped in oligomannose, hybrid or complex type, the latter being the most abundant (about 90%) in IgG, either circulating or produced by cell lines *in vitro* (25) (**Figure 1A**). The presence of Fc N-glycan induces in general a more open structure compared to aglycosylated IgG, favors binding to activating FcγRs and promotes antibody stability *in vitro* and *in vivo* (1, 26). The complex type N-glycosylation itself shows microheterogeneity: whereas it always contains a heptaglycan biantennary core structure (four GlcNAc and three mannose residues), the core can bear an additional bisecting GlcNAc (in about 10% of IgGs), and 1 or 2 galactose residues (in 35% and 15% of IgG, respectively) and 1-2 terminal N-acetylneuraminic acid (sialic acid, SA), on 10-15% of IgGs (**Figure 1B**). Finally, an α-1,6 fucose residue (core fucose) is present in 90% of complex type IgG N-glycans (**Figure 1B**). Interestingly the presence of bisecting GlcNAc inhibits α-1,6 core fucosylation due to steric hindrance and therefore IgGs generally contain either a bisecting GlcNAc or a core fucose residue, although some IgGs may have both bisecting GlcNAc and fucose (2, 27–29). IgGs are composed of least 30 glycovariants, to which specific abbreviations have been assigned: G0 (no Gal residue), G1 (1 Gal), G2 (2 Gals), F (fucose) etc (**Figure 1B**) (2, 27–29).

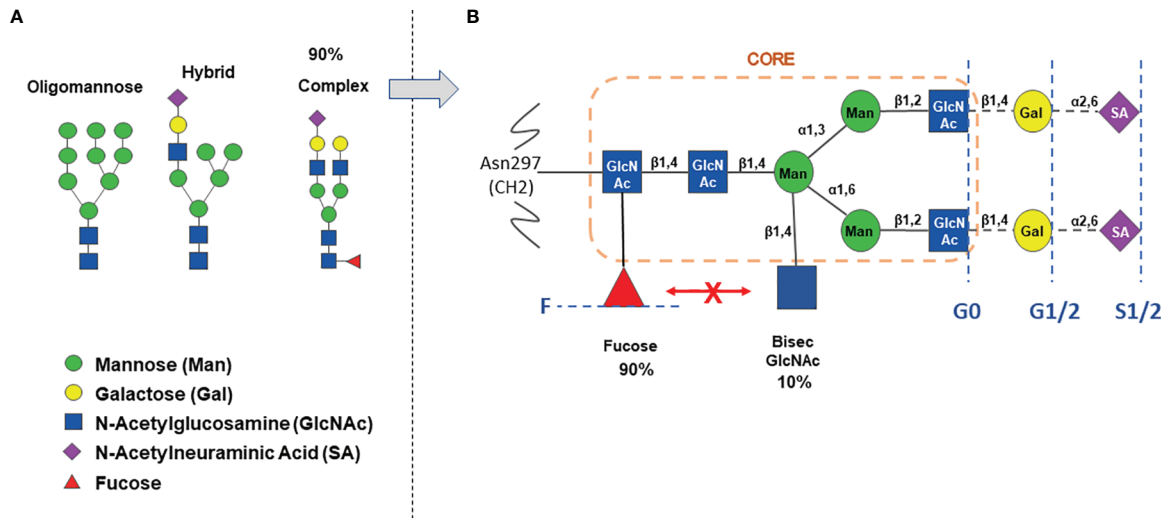

FIGURE 1 | The IgG Asn-297 N-glycan heterogeneity. Panel **(A)** major types of N-glycosylation observed in IgG. Panel **(B)** detail of complex type glycosylation with percentage of circulating IgG containing either fucose or bisecting N-Acetylglucosamine. The orange circle indicates the core structure. The blue broken lines and text indicate the heterogeneity of complex N-glycans, carrying either no Gal (G0), 1-2 Gal (G1/2), 1-2 Sialic acids (S1/2), with (F) or without core α-1,6-fucose.

THE BIOSYNTHESIS OF HUMAN IGG N-LINKED GLYCANS

N-glycosylation is a multi-step enzyme-mediated biochemical process. IgG N-glycan biosynthesis starts in the endoplasmic reticulum with the addition of a pyrophosphate-dolichol precursor (Dol-P, Glc3Man9GlcNAc2) to the Asn 297 N-glycosylation site of IgG. This structure is then trimmed by glucosidases and mannosidases, as the process moves to the Golgi, leading to the formation of high mannose, hybrid and the complex types N-glycans (26, 30–33). The main enzymatic reactions taking place in the Golgi are summarized in **Figure 2**. The stable overexpression or reduction/inhibition of some of these enzymes in antibody producing cell lines have been used to modify IgG glycan composition and perform structure-function studies of N-glycan microheterogeneity ((31); and see below). The structure of N-linked and other IgG glycans and glycosylation pathways have already been extensively reviewed by others (30, 32, 34, 35) and we refer the readers to these more complete descriptions of the process.

The enzymatic pathways for GDP-fucose biosynthesis and Fc core fucosylation are shown in **Figure 3** (26, 30, 36). L-Fucose (6-deoxy-L-galactose) is a monosaccharide obtained by glycoprotein degradation or diet. In the salvage pathway, L-fucose is phosphorylated in the cytosol to fucose-1-phosphate by fucokinase (FUK), and then converted to GDP-fucose by GDP-fucose-pyrophosphorylase (GDPP), an essential substrate for the fucosylation of proteins (**Figure 3**). Alternatively, and most commonly, *de novo* GDP-fucose is synthesized from GDP-mannose by GDP-mannose 4,6 dehydratase (GMD) and then GDP-4-keto 6-deoxymannose 3,5-epimerase-4-reductase (FX) (**Figure 3**). GDP-fucose is transported to the ER *via* the SLC35C1 and SLC35C2 transporters and used by several fucosyltransferases (FUT) in the Golgi to fucosylate glycoproteins. There are 11 different FUTs, but only FUT 8 catalyzes IgG Fc core fucosylation *via* an α-1,6 linkage (**Figure 3**). As mentioned above, the presence of bisected GlcNAc inhibits the addition of core α-1,6 fucose (26, 30, 36).

These pathways show that there are multiple steps where a modulation of fucosylation can take place including the supply of diet fucose (36). The mechanisms of IgG Fc glycosylation regulation in B cells in healthy individuals and in disease are however still little understood.

STRATEGIES TO GENERATE THERAPEUTIC IGG1 WITH LOW LEVELS OF FUCOSE LEVELS

In the last 10-15 years, removal of IgGs Fc core fucose has been shown to be an important method to enhance ADCC by therapeutic IgG1 mAbs (37–39). Based on the knowledge of the pathways of N-glycan fucosylation described above, several strategies have been used to generate therapeutic IgGs with low or no fucose [reviewed by Pereira et al. (40)]. These are briefly summarized below:

i. The selection of cell lines that naturally have low FUT8 enzyme, such as the Y2B/0 rat cell line (38).

ii. The creation of FUT8 knock out cell lines such as CHO FUT8$^{-/-}$ (41, 42). These produce antibodies completely lacking fucose, but may have a low growth rate unless specifically adapted.

iii. The selection of natural cell lines with defects in other enzymes involved in fucosylation, such as Lec 13, which has defective GDP mannose 4,6 dehydratase (GMD) which converts

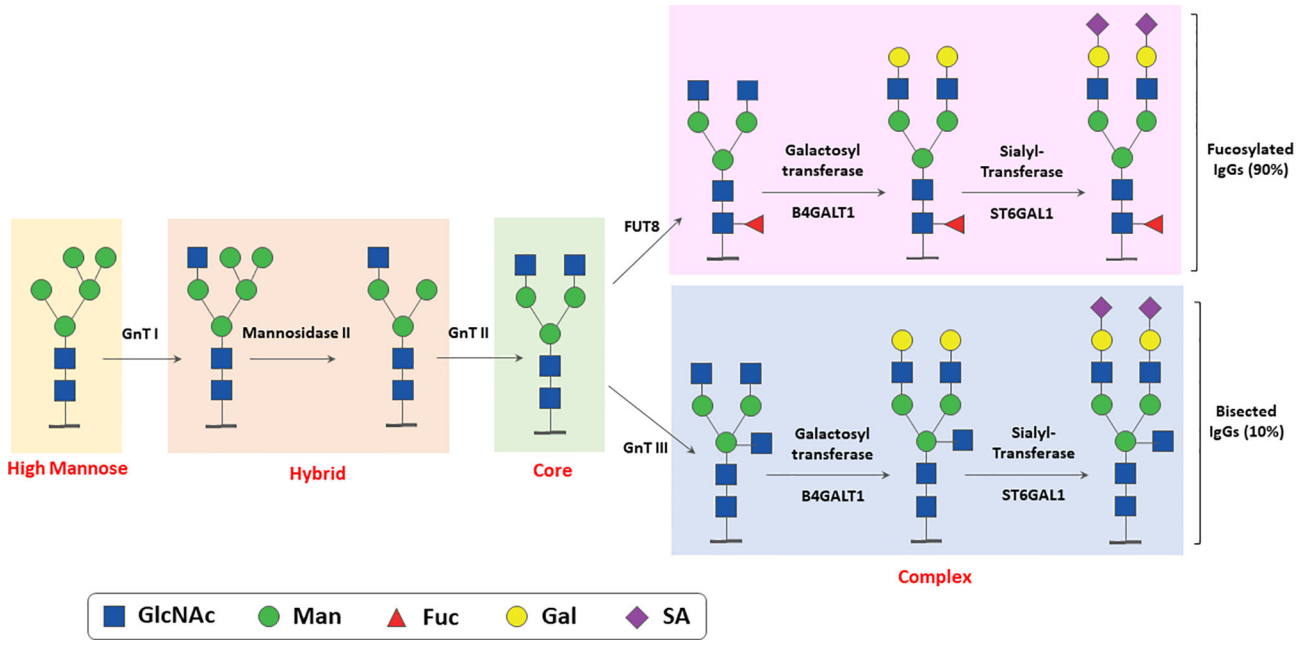

FIGURE 2 | Simplified scheme of enzymatic reactions involved in N-linked glycosylation of IgG. The major glycosylation steps and enzymes involved in N- linked glycosylation of IgGs taking place in ER and Golgi are shown. GnT, N-Acetyl glucosamine transferase; FUT, Fucosyl transferase; Man, Mannose; Gal, Galactose; GlcNAcm N-acetylglucosamine; SA, sialic acid (N-Acetylneuraminic Acid).

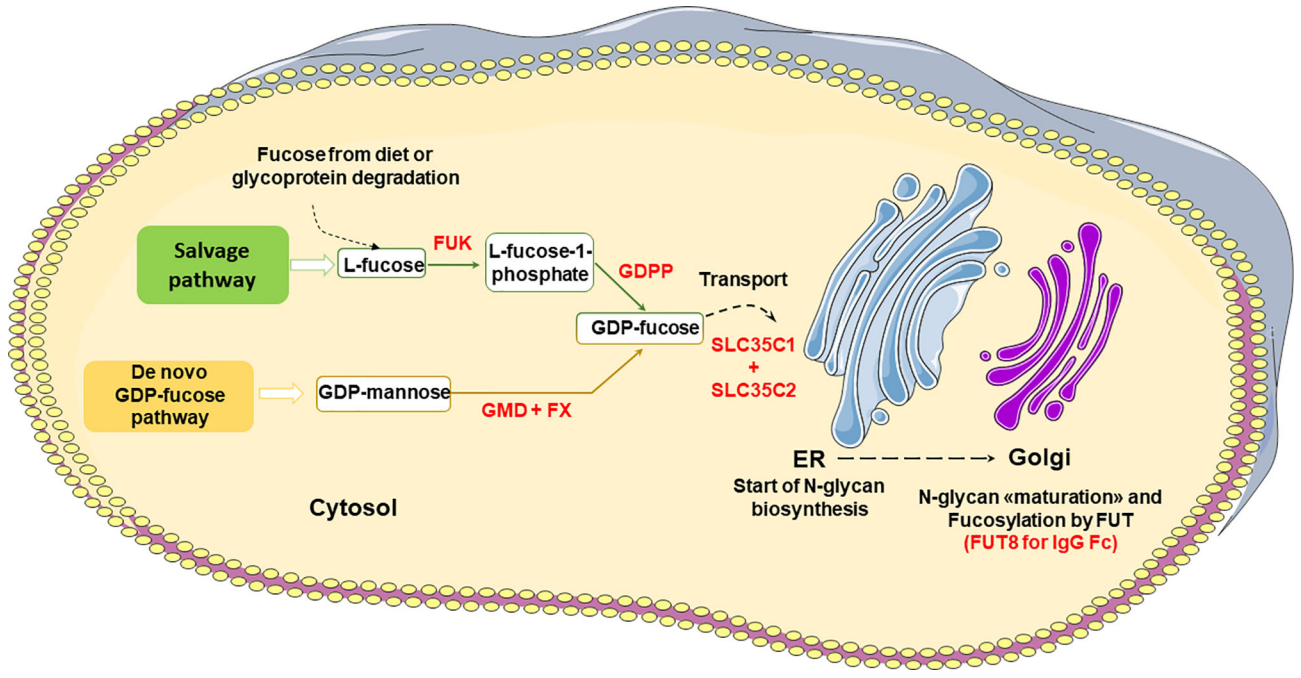

FIGURE 3 | Major pathways of GDP-fucose biosynthesis and IgG Fc core fucosylation. FUK, Fucose Kinase; GDPP, GDP-fucose-pyrophosphorylase; GMD, GDP-mannose 4,6 dehydratase; FX, GDP-4-keto 6-deoxymannose 3,5-epimerase-4-reductase; FUT, Fucosyltransferase.

GDP mannose to GDP-fucose, the substrate for N-glycan core fucosylation (**Figure 3**) (37, 43).

iv. The creation of engineered cell lines lacking GMD, GDP-L-fucose synthase (FX) and/or the GDP-fucose transporter SCL35C1 (**Figure 3**) (44, 45). For example, the FX$^{-/-}$ and GMD$^{-/-}$ CHOZN® cell clones produced IgG1 antibody with core fucosylation reduced to 6-8% or 1-3%, respectively. The FX$^{-/-}$ clones also showed some aberrant glycan forms suggesting the GMD$^{-/-}$ CHOZN® cell line may be the best choice (44).

v. Another strategy is to engineer CHO clones with higher β-1,4-N-acetylglucoseaminyl-transferase III (GnTIII), best with a Golgi localization domain (2, 31, 46, 47). GnTIII catalyzes the transfer of GlcNAc to a core mannose residue in N-linked oligosaccharides *via* a β-1,4 linkage, which results in the formation of a bisected sugar chain. The bisection GlcNAc inhibits the transfer of fucose by FUT8, so that GnTIII overexpressing cells produce antibodies with lower levels of core fucose.

vi. Another modality is to use non-mammalian cells such as plant or insect cells that are engineered to synthesize human-type N-glycans, to reduce potential immunogenicity, but lack core fucosylation capacity (48).

vii. Cells can be treated with inhibitors of FUT8 such as 2F-Peracetyl-Fucose (49) or anti-FUT8 antibody (50).

viii. Finally, antibodies can also be enzymatically modified *in vitro* by treatment with glycosidase and glycosynthase enzymes. This is somewhat more complex since it requires antibody deglycosylation followed by a controlled glycosylation (51, 52).

The major strategies based on engineered mammalian cell lines are listed in **Table 1**.

It is worth noting that, depending on the system used, the amount of fucose may vary from 0% for cell lines lacking FUT8 enzyme to 10-30% for the those with reduced FUT8 or other enzymatic modifications (**Table 1**). Also, different production cell lines and systems may lead to antibodies with different N-glycan profiles, not only regarding fucose residues. These may in turn affect function of antibodies since carbohydrates other than fucose, for example galactose or sialic acid may affect CDC or inflammation, respectively, among others (33, 53). A more detailed description of the role of galactosylation and sialic acid is beyond the scope of this review.

FUNCTIONAL CONSEQUENCES OF LOW CORE FUCOSE ON IGG1 ASN 297 N-GLYCAN

Binding to FcγRIIIA and FcγRIIIB

Human, humanized or chimeric IgG1 antibodies lacking fucose on the Asn N-glycan bind with 10-100 fold higher affinity to human FcγRIIIA and FcγRIIIB (CD16B) (37, 54, 55). Structural studies have shown an increased carbohydrate-carbohydrate interaction between the N-glycans of FcγRIIIA and Fc, explaining the higher affinity of afucosylated IgG1 (56, 57).

NK Cells and ADCC

Since FcγRIIIA is the major activating receptor on NK cells and mediates ADCC, the net result of increased binding to FcγRIIIA is a significant enhancement of ADCC by afucosylated IgG1 antibodies with respect to their fully fucosylated counterpart (2-40 fold, also depending on galactosylation) (37–39) (**Table 2**). In addition, FcγRIIIA has relatively low-medium affinity for IgG so that ADCC is inhibited by excess IgG in plasma. In contrast, ADCC induced by afucosylated IgG1, which has a significantly higher affinity for FcγRIIIA, is not significantly inhibited by plasma IgG. Thus afucosylated anti-CD20 antibody may be more effective in inducing ADCC in whole blood by 2 mechanisms: 1) higher affinity for FcγRIIIA and 2) significantly reduced inhibition by serum IgG (99). Afucosylated IgG1 has also been reported to induce greater FcγRIIIA downmodulation from the NK cell surface, a phenomenon which takes place *via* shedding of the extracellular domain by the ADAM 17 metalloproteinase and may participate in the serial target cell killing by NK cells (100).

Phagocytosis by Macrophages

FcγRIIIA is expressed by monocytes/macrophages, particularly M2 and red pulp macrophages as well as microglial cells. However, macrophages also express the activating FcγRI and FcγRIIA, and FcγRI is thought to be the major mediator of phagocytosis of IgG1 opsonized targets (10, 11, 101). Therefore, afucosylated therapeutic mAbs, despite binding with higher affinity to FcγRIIIA, are not generally reported to significantly enhance phagocytosis, at least *in vitro* (10, 11, 102). There is some evidence that phagocytosis of targets opsonized with anti-

TABLE 1 | Examples of cell lines and strategies developed for low fucose antibody production.

Cell line or system	Enzyme defect	Approximate Fc core fucosylation level (normal is >90%)	References
Lec 13 (CHO mutant)	Defective GMD	10%	(37, 43)
YB2/0 (rat)	Low FUT 8	9-30%	(38)
CHO FUT8$^{-/-}$ (e.g. Potelligent®)	FUT8 KO	0%	(41, 42)
CHO GMD$^{-/-}$GFT$^{-/-}$	GMD+SLC35C1 KO	0%	(45)
CHO FX$^{-/-}$	FX KO	6-8%	(44)
CHO GMD$^{-/-}$	GMD KO	1-3%	(44)
CHO GnTIII^{+++} (e.g. GlycomAbs®)	GnTIII overexpression	10-15%	(46, 47)

FUT, Fucosyltransferase; GMD, GDP-mannose 4,6 dehydratase; FX, GDP-4-keto 6-deoxymannose 3,5-epimerase-4-reductase; GnTIII, N-acetylglucosamine transferases III; SLC35C1, GDP-fucose transporter; KO, knock out.

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