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REVIEW ARTICLE

Improving Antibody-Based Cancer Therapeutics Through Glycan Engineering

Xiaojie Yu[1] · Michael J. E. Marshall[1] · Mark S. Cragg[1] · Max Crispin[2,3]

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Abstract Antibody-based therapeutics has emerged as a major tool in cancer treatment. Guided by the superb specificity of the antibody variable domain, it allows the precise targeting of tumour markers. Recently, eliciting cellular effector functions, mediated by the Fc domain, has gained traction as a means by which to generate more potent antibody therapeutics. Extensive mutagenesis studies of the Fc protein backbone has enabled the generation of Fc variants that more optimally engage the Fcγ receptors known to mediate cellular effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and cellular phagocytosis. In addition to the protein backbone, the homodimeric Fc domain contains two opposing N-linked glycans, which represent a further point of potential immunomodulation, independent of the Fc protein backbone. For example, a lack of core fucose usually attached to the IgG Fc glycan leads to enhanced ADCC activity, whereas a high level of terminal sialylation is associated with reduced inflammation. Significant growth in knowledge of Fc glycosylation over the last decade, combined with advancement in genetic engineering, has empowered glyco-engineering to fine-tune antibody therapeutics. This has culminated in the approval of two glyco-engineered antibodies for cancer therapy: the anti-CCR4 mogamulizumab approved in 2012 and the anti-CD20 obinutuzumab in 2013. We discuss here the technological platforms for antibody glyco-engineering and review the current clinical landscape of glyco-engineered antibodies.

Key Points

Antibody glycosylation can significantly influence clinical efficacy.

Design and selection of antibody glycoforms offers a route to enhanced therapies.

1 Introduction

Since the approval in 1997 of rituximab, the first monoclonal antibody (mAb) approved for the treatment of cancer, antibody-based therapies have revolutionised the field of clinical oncology [1, 2]. The success of rituximab has driven an explosion of interest in antibody therapeutics and fostered the desire to identify other therapeutic targets and to augment antibody efficacy through protein engineering [3]. As of June 2016, 24 mAbs, mostly human IgG-based, had been approved by the US Food and Drug Administration (FDA) for the treatment of solid or haematological tumours [4–6].

Antibodies used in cancer treatment can be broadly classified into two categories based on their target type. The conventional ‘direct targeting’ class of cancer

✉ Xiaojie Yu
x.yu@soton.ac.uk

✉ Max Crispin
max.crispin@bioch.ox.ac.uk

[1] The Antibody and Vaccine Group, Cancer Sciences Unit, University of Southampton, Faculty of Medicine, General Hospital, Southampton SO16 6YD, UK

[2] Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

[3] Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA 92037, USA

antibodies, comprising the majority of approved antibodies such as the anti-CD20 rituximab and anti-Her2 trastuzumab, target tumour cells by direct engagement of either lineage-specific antigens (e.g. CD20), tumour neoantigens (e.g. glycans) or overexpressed oncogenic antigens [e.g. epidermal growth factor receptor (EGFR)]. After antibody engagement, the tumour cells are selectively depleted via various routes such as signalling-induced apoptosis or Fc-mediated complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC) and/or antibody-dependent cell-mediated phagocytosis (ADCP), depending on the nature of the antibody, target antigen and epitope [7]. The second class of anti-cancer antibodies, so-called immunomodulatory mAbs, do not directly engage the tumour cells but rather target receptors on cells of the host immune system in an attempt to stimulate increased activity, principally through cytotoxic $CD8^+$ T cells [8–10]. Antibody immunotherapy of this type came to the spotlight when the first immune 'checkpoint blocker' anti-CTLA4 mAb ipilimumab received approval to treat advanced melanoma, following the demonstration that it could significantly improve the prognosis for a proportion of patients with this aggressive malignancy [11].

Despite the evident clinical efficacy of anti-cancer antibodies, their modes of action are often incompletely understood. It is generally accepted that for the conventional direct-targeting antibodies, the variable domain targets the tumour-selective antigen, conferring specificity, while the Fc domain mediates the elimination of tumour cells via the host immune system [12, 13]. However, antibodies designed to effectuate through the blockade of signal transduction, such as the anti-Her2 trastuzumab, may also work in part through the same mechanisms and moreover could attain additional functionality through Fc engineering. The relative contribution of the variable and Fc domains to the immunomodulatory mAbs is currently unclear. For example, agonistic anti-CD40 mAbs, which stimulate host antigen-presenting cells to potentiate an immune response against the tumour by activating CD40, a tumour necrosis factor receptor (TNFR), were also found to require the Fc domain for therapeutic efficacy [14]. However, the specific Fcγ receptor (FcγR) required was shown to be entirely different to that engaged by direct targeting mAb in several pre-clinical models [14–18]. Moreover, isotype switching to the less common IgG2 obviated the need for the Fc domain, resulting in Fc-independent agonistic activity [15, 19]. Similar paradigms have been seen with other TNFR targets, demonstrating that the Fc and its interaction with FcγR are critical in delivering therapeutic responses for many antibody classes [15, 20].

2 Rationale for Antibody Glyco-Engineering

2.1 The Fc-FcγR Engagement

The general requirement of the IgG Fc domain for a fully functional cancer antibody has prompted the use of Fc engineering to improve the efficacy of mAb effector functions. The IgG Fc engages a set of FcγRs that are expressed predominantly on immune effector cells to mediate effector functions [21, 22]. These FcγRs are categorised as either activatory or inhibitory based on their intracellular signalling motif. The human activatory FcγRs (FcγRI, FcγRIIa, FcγRIIc and FcγRIIIa) contain an intracellular activatory immunoreceptor tyrosine-based activation motif (ITAM) signalling motif within the cytoplasmic domain of the receptor (FcγRIIa and IIc) or on the associated FcγR chain; while the sole inhibitory FcγRIIb contains an inhibitory immunoreceptor tyrosine-based inhibitory motif (ITIM) within its cytoplasmic domain [22, 23]. Humans additionally express another FcγR, FcγRIIIb, which is highly homologous to FcγRIIIa in its extracellular domain but contains no transmembrane or intracellular regions, instead being anchored to the membrane through a glycophosphoinositol anchor and expressed solely on neutrophils [24]. Mice express four homologs, three of which (FcγRI, FcγRIII and FcγRIV) are activatory while FcγRIIb is inhibitory, as in humans [25].

In humans, the activatory FcγRIIIa is widely regarded to be the most important for delivering natural killer (NK) cell-mediated ADCC, as FcγRI and IIa are not expressed on these cells and FcγRIIc is only expressed in a relatively small proportion of people due to the presence of a stop codon [26]. Furthermore, a functional polymorphism present within FcγRIIIa at position 158, which alters the receptor's affinity for IgG1 and 3, has been reported to significantly affect the clinical response to rituximab therapy [27, 28]. The importance of effective Fc-FcγR engagement for anti-tumour antibody efficacy has led to exhaustive Fc mutagenesis studies, which have identified residues critical for FcγR engagement and also enabled the generation of Fc variants that selectively engage specific FcγRs [29]. Many of these characterisations were carried out in vitro in systems where the mAb of interest represents the dominant antibody, which fails to recapitulate the in vivo environment where a high concentration of endogenous serum IgG (approximately 10 mg/mL) will compete with exogenously administered therapeutic antibodies for FcγR engagement [30]. Weakening such intrinsic endogenous IgG-FcγR interaction through the inactivation of serum IgG Fc, a strategy termed 'receptor refocusing', could effectively improve the activity of therapeutic mAbs [31].

2.2 Antibody Glycosylation and FcγR Engagement

Besides protein mutagenesis, antibody Fc glycosylation could also significantly modulate the Fc-FcγR interaction, through its conserved N-linked oligosaccharide attached to asparagine 297 [32]. Antibody N-glycosylation occurs co-translationally in the lumen of the endoplasmic reticulum (ER) and initially follows a highly conserved and well delineated pathway involving the transfer of a large pre-assembled glucosylated oligomannose-type oligosaccharide. As the protein transits from the ER and through the Golgi apparatus, different glycan transferases and glycosidases add or remove different monosaccharides to create a diverse range of oligosaccharides differing in size and complexity. The diversity of IgG Fc glycoforms, like the myriads of other glycoproteins, arises from both the cellular environment and the intrinsic three-dimensional structure of the protein. However, despite the potential for considerable chemical heterogeneity, IgG Fc glycosylation is characterised by a predominant population of glycans displaying limited processing of terminal residues giving rise to a characteristic fingerprint of glycoforms with partial galactosylation.

Our current understanding of how the Fc glycan regulates antibody function has undergone a recent transition from being a simple structural support to a major immunomodulating agent. Early evidence supporting the importance of Fc glycan came from in vitro studies where IgG deglycosylation abrogated its effector functions [33–35]. Since then, divergent activities of various IgG glycoforms, whether naturally occurring or engineered, have firmly established its immune-modulatory potential. The first clinical association was noted in rheumatoid arthritis patients, where a lack of terminal galactose in the IgG Fc glycoforms was correlated with disease induction, suggesting a pro-inflammatory role of agalactosylated glycoforms [36]. This was further supported by reports that the typical period of symptom remission noted during pregnancy was associated with a decrease in the level of agalactosylated IgG glycans [37–39]. Furthermore, HIV controllers who demonstrate better viral containment exhibit globally more agalactosylated IgG glycoforms [40]. In contrast to agalactosylation, more recently, it was described that Fc bearing terminal α2,6-linked sialic acids possess general anti-inflammatory effects in vivo [41, 42] and this effect may be influenced by the fucosylation status [43].

Besides galactose and sialic acid, the monosaccharide that has the most explicit influence on antibody therapeutics is the core fucose: fucose α1,6-linked to the protein-proximal GlcNAc residue. Engineered IgG Fc that lacks the core fucose was initially found to display significantly enhanced ADCC through improved engagement with FcγRIIIa, an observation since reproduced by many independent laboratories [44–48]. The crystal structures of fucosylated Fc in complex with the human FcγRIIIa elegantly illustrate how the receptor glycan clashes with the antibody fucose; a conflict which is resolved with afucosylated Fc, explaining its enhanced affinity [48, 49] (Fig. 1).

2.3 Fc Engineering and Immunogenicity

The human immune system is well known for its ability to recognise and reject foreign entities, through both humoral and cell-mediated responses. Therefore, it is not surprising that, for example, patients receiving the chimeric mouse/human anti-CD20 rituximab can elicit a human anti-mouse antibody immune response directed against the remaining mouse framework regions upon first exposure, which then complicates repeated use due to hypersensitivity reactions and reduced mAb half-life [50, 51]. Site-directed mutagenesis of native antibody Fc domains, which dominates the current landscape of Fc engineering, could therefore potentially spawn Fc neo-immunogens, even though there is a lack of such reports in the current literature. On the contrary, Fc glyco-engineering, predicated on the appendage of defined endogenous glycoforms, may in fact circumvent immunogenicity.

Nevertheless, for mAbs produced in non-human cell lines, the impact of glycosylation on antibody immunogenicity has been recognised as a potential issue. For example, the anti-EGFR cetuximab, which contains both Fab and Fc N-linked glycans, was found to contain the non-human glycan structure galactose-α1,3-galactose in its Fab glycans when produced in mouse Sp2/0 cells [52, 53]. Humans express natural IgG reactive towards galactose-α1,3-galactose, and it was discovered that patients with high levels of pre-existing anti-galactose-α1,3-galactose IgE, a class typically associated with allergy, displayed a hyper-sensitivity reaction to cetuximab [54, 55]. Interestingly, the same cetuximab-specific IgE antibodies isolated from patients that had demonstrated hypersensitivity did not react with cetuximab produced in Chinese hamster ovary (CHO) cells which naturally lack α1,3-galactosyltransferase activity. This further highlights the importance of antibody glycosylation and the choice of appropriate host cell line for therapeutic antibody production.

Given the immunomodulatory capacity of Fc glycan, glyco-engineering presents a viable path besides protein engineering to optimise the therapeutic activity of anti-tumour antibodies. As fucose engineering dominates the current portfolio of tumour-targeting antibodies, we focus this review on platforms for fucose engineering, and discuss fucose-engineered antibodies. It is, however, important to note that other means of glyco-engineering could prove equally useful in the future, especially for the

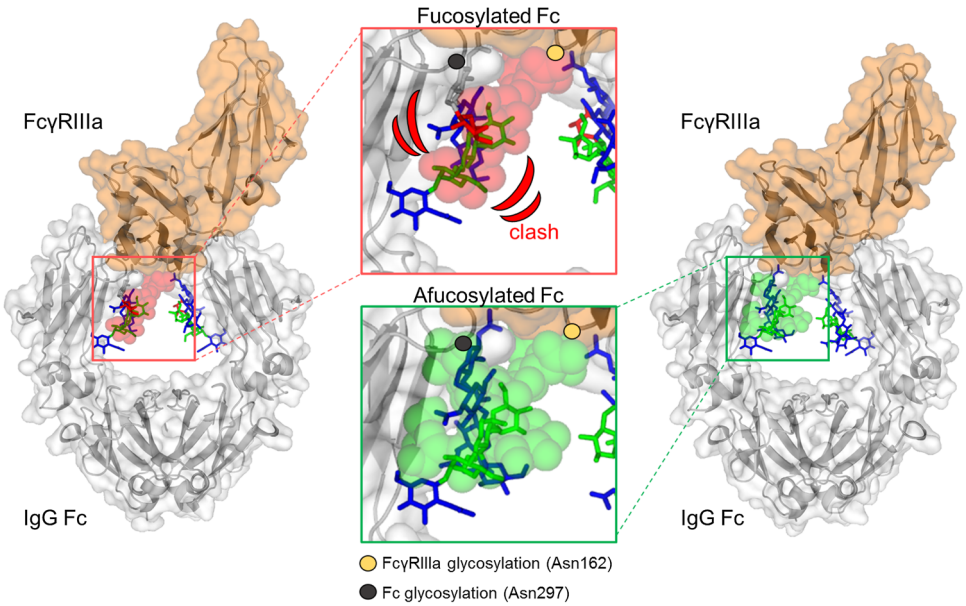

Fig. 1 Structural basis of afucosylation-mediated enhancement of IgG receptor interaction. Crystal structures of fucosylated and afucosylated IgG1 Fc in complex with FcγRIIIa containing a glycan at Asn162 reported by Ferrara et al. [48]. The FcγRIIIa component is shown in *orange* with transparent surface representation and cartoon representation of the protein chain. The FcγRIIIa glycan at Asn162 is shown in either *red* or *green* for the structure containing Fc fucose and that without Fc fucose, respectively. The Fc protein is shown in *grey* with transparent surface and cartoon representation of the protein chain. Fc glycans are shown as sticks. The steric clash between the receptor glycan and the Fc fucose is highlighted by *cartoon clash symbols*

immunomodulatory antibodies whose glycoforms remain largely unexplored.

3 Platforms for Fucose Engineering

IgG Fc with a low level of core fucose engages FcγRIIIa and FcγRIIIb with higher affinity and therefore leads to enhanced ADCC and ADCP [56–58]. This finding had triggered extensive research into methods for generating low fucose or fully afucosylated IgG. Several major methodologies have emerged to be applicable in making therapeutic antibodies.

3.1 Genetic Modification of the Host Biosynthesis Pathway

Altering the endogenous host N-glycosylation pathway could either reduce or completely remove fucose from antibody Fc. In a landmark study by Umana and colleagues [58], it was discovered that overexpressing the GnTIII enzyme, which catalyses the addition of bisecting GlcNAc, in CHO cells led to the production of recombinant IgG1 mAb with significantly reduced core fucosylation, associated with higher ADCC in vitro. The technology based on this GnTIII overexpression for generating low fucose therapeutics is referred to as GlycoMab, which was acquired by Roche [59]. The addition of bisecting GlcNAc to Fc glycan by GnTIII pre-empts the downstream action of the α1,6-fucosyltransferase; thus, bisecting GlcNAc acts as a competitive inhibitor of core fucosylation, and overexpression of GnTIII in mammalian cells provides a means of producing IgG with low fucose [60]. To further diminish the fucose level, the same group engineered a recombinant form of GnTIII, which possesses the localisation motif of the Golgi α-mannosidase II, whose overexpression gave rise to approximately 60–70% afucosylated complex and hybrid-type glycans [61]. As we will discuss later, this GnTIII-engineered platform forms the basis of producing obinutuzumab, the recently approved anti-CD20 mAb used for the treatment of non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukaemia (CLL). While the impact of bisecting GlcNAc predominantly impacts ADCC through its inhibitory effect on core fucosylation, there are some indications that GlcNAc might modulate receptor affinity independent of this effect [62].

Although the overexpression of GnTIII in a cell line leads to reduced levels of fucose, this is not complete and there remains significant room for improvement, that is, complete defucosylation. One means of achieving this involves genetically disabling the α1,6-fucosyltransferase encoded by the *FUT8* gene. Just such a *FUT8* knock-out (KO) cell line was generated in CHO cells via homologous recombination, and led to the production of 100% afucosylated antibodies [63]. This *FUT8* KO antibody production platform was coined Potelligent technology, and was

the system used to develop the anti-Cysteine-Cysteine chemokine receptor 4 (CCR4) mAb, mogamulizumab, the first approved glyco-engineered antibody [64] (see Sect. 4.1). Another means of dampening α1,6-fucosyltransferase activity involves siRNA targeting of *FUT8*. Unfortunately, these reagents are not highly efficient, reducing *FUT8* transcription by only 20%, resulting in approximately 60% afucosylated IgG [65].

Apart from a direct *FUT8* gene KO, a second approach involves interfering with the GDP-fucose de novo biosynthesis pathway, whose resulting substrate GDP-fucose is essential for routinely cultured cells to produce fucosylated glycoproteins. One target enzyme in the pathway, the GDP-mannose 4,6-dehydratase (GMD), is involved in the catalytic process of converting D-glucose to GDP-fucose, the substrate of α1,6-fucosyltransferase that is indispensable for IgG core fucosylation [66]. CHO cells deficient in the GMD gene showed a complete lack of GDP-fucose, and in the absence of exogenous L-fucose produced 100% afucosylated IgG [67]. Yet another means of interfering with GMD activity involves the heterologous expression of the GDP-6-deoxy-D-lyxo-4-hexulose reductase (RMD). RMD catalyses the production of monosaccharide GDP-D-rhamnose, which provides feedback inhibition to GMD activity [68], resulting in less GDP-fucose production and lowered antibody fucosylation [69]. The introduction of heterologous RMD produced a surprisingly high (98%) level of afucosylated IgG, reflecting the potency of this feedback inhibition pathway [69]. The technology based on this heterologous induction of RMD is referred to as GlymaxX, developed by the company ProBioGen.

The above-mentioned glyco-engineering methods, notably carried out in CHO cells, produce the most mammalian-like IgG glycoforms comprising mostly biantennary complex-type glycans. Oligosaccharides early in the N-glycosylation pathway intrinsically lack core fucose, as the α1,6-fucosyltransferase only acts from the mid-phase of the N-glycosylation pathway with the target glycans becoming more favoured substrates following the action of GnTI [70]. Therefore, retaining the oligosaccharide in its early immature form, prior to GnTI action, should enable the production of relatively homogenous afucosylated glycoforms. Indeed, the HEK293S cell line, which is engineered to be deficient in the GnTI enzyme required for oligosaccharide maturation, is commonly used to produce afucosylated oligomannose glycoforms [71, 72]. However, despite homogeneous afucosylation, the predominance of the oligomannose antibody glycoforms produced deviates significantly in composition from the endogenous biantennary complex-type glycoforms [73], triggering debates over its perceived inferior in vivo pharmacokinetics [74–76]. On balance, it seems that oligomannose-type glycoforms of IgG do exhibit more rapid serum clearance, presumably driven by lectin-mediated clearance pathways [75]. Nonetheless, this HEK293S cell line remains a useful glyco-engineering tool in the laboratory.

Besides mammalian cells, other eukaryotic systems have been engineered to produce low fucose antibodies. For example, the yeast *Pichia pastoris* has been extensively engineered to produce both homogeneous fucosylated and non-fucosylated glycoforms [77]. More recently, the unicellular ciliate, *Tetrahymena thermophile*, has been reported to generate highly afucosylated mAbs with the advantageous ease of production [78]. Furthermore, the plant *Lemna minor*, when transfected with siRNA to suppress its intrinsic α1,3-fucosyltransferase and β1,2-xylosyltransferase activity, was able to generate 96% afucosylated, di-galactosylated glycoforms [79]. Notwithstanding the versatility of such non-mammalian systems, the existence of non-self carbohydrate epitopes, even in minute amount, has generally been considered a source of concern for their clinical use.

3.2 Metabolic Interference of Host Biosynthesis Pathway

Genetic modification of cellular systems usually requires significant investment, both temporal and financial. There are also substantial regulatory hurdles to pass before new cell lines are approved for the production of clinical grade material. Metabolic interference, which employs soluble enzyme inhibitors, potentially provides an alternative to the generation of new cell lines and is especially useful in preclinical laboratory settings where proof of concept takes priority. One molecule, kifunensine, an alkaloid inhibitor of the α1,2-mannosidase I enzyme, prevents the mannose trimming of the initial oligosaccharide and thus retains the N-glycan in its early afucosylated oligomannose form [80, 81]. Moreover, synthetic inhibitors (such as 2-fluorofucose) based on the structure of metabolic intermediates of the GDP-fucose de novo synthesis pathway were shown to elicit 95% afucosylated glycoforms in CHO cells [82]. However, compared with genetically modified host cell lines, inhibitor-based metabolic interference can prove impractical when production is scaled up for clinical use.

3.3 Post-Translational Enzymatic Modification

An alternative strategy involves employing post-translational modification. Rapid advancement in chemical biology has expanded the set of synthetic tools available for post-translational glycan modification. In recent years, chemoenzymatic glyco-engineering has emerged as a reliable method for generating homogeneously glycosylated antibodies. In vitro enzymatic or chemoenzymatic glyco-engineering involves serial enzymatic treatments of

purified antibodies to achieve the desired glycoform, which offers the potential to produce both diverse yet homogeneous glycoforms. For example, the serial treatment of IgG with recombinant galactosyltransferase and sialyltransferase coupled with their respective substrate is able to generate tetra-sialylated antibodies with an anti-inflammatory effect [41]. However, much work still remains to do in this field as certain enzymatic reactions, such as defucosylation, within the N-glycosylation pathway cannot yet readily be reproduced in this manner on natively folded antibodies with the full biantennary complex glycans [83].

Therefore, transglycosylation, a method in which the bulk Fc oligosaccharide is first removed followed by the addition of pre-synthesised glycans of the desired structure, was developed to bypass some inefficient glycosyltransferases and glycosidases in the N-glycosylation pathway. The first-generation transglycosylation was pioneered by the Wang group that uses EndoH to deglycosylate the bulk oligomannose Fc glycan, followed by the addition of synthetic small biantennary glycans back onto the same glycosylation site using the glycosyltransferase EndoA to generate fully afucosylated Fc glycoforms [62]. However, EndoA lacks the ability to transglycosylate the typical full-length complex-type glycans, which prompted the engineering of another bacterial glycosidase EndoS. EndoS usually deglycosylates Fc glycan but when mutated could be converted into a versatile Fc-specific trans-glycosyltranferase [83]. Indeed, the serial application of wild-type EndoS to first remove the bulk Fc glycan and the fucosidase to remove any Fc core fucose, followed by the mutated EndoS (trans-glycosyltranferase) for the addition of fully afucosylated glycan, generated a human-like afucosylated Fc glycoform [83]. It is important that glycans pre-synthesised for transglycosylation in vitro do not undergo autocatalysis such as epimerisation, which has been reported to contribute to heterogeneity [84]. Thus, chemoenzymatic glyco-engineering is an emerging route to generate fully afucosylated Fc glycoforms. However, this approach is likely to be advantageous over simple cell line or metabolic engineering mainly when particular synthetic challenges are faced; for example, when transglycosylation is employed to generate glycan-based antibody–drug conjugates [85]. Moreover, transglycosylation presents a significant challenge to cost of goods, and is likely to be highly impractical as a manufacturing process unless it imparted critical functionality that could be obtained no other way.

4 Glyco-Engineered Antibodies in Clinical Oncology

Most antibodies approved for cancer therapy have demonstrated, to various extents, components of ADCC or ADCP in their therapeutic efficacy. Afucosylated antibody glycoforms uniquely exhibit enhanced Fc engagement with the activatory FcγRIIIa and FcγRIIIb resulting in more potent ADCC and ADCP [56–58]. Since the first definitive report of defucosylation-mediated ADCC enhancement in the early 2000s [44], two glyco-engineered, afucosylated mAbs have already been approved for cancer therapies [86, 87]. A list of fucose-engineered antibodies developed for clinical oncology is shown in Table 1.

It is important to note that while fucose-engineered mAbs have gained most attention for cancer indications, various glycan-engineered mAbs are also being developed for other diseases. For example, roledumab, a low fucose anti-Rhesus D mAb, is currently in a phase II/III clinical trial to combat haemolytic disease of the foetus or newborn [6, 88]. This section will focus on glyco-engineered antibodies for cancer treatment.

4.1 Mogamulizumab, the First Approved Glyco-Engineered mAb

As detailed above, mogamulizumab was the first approved (in Japan) glyco-engineered mAb for cancer therapy [89]. Generated through the Potelligent technology, it is an afucosylated therapeutic targeting the chemokine receptor CCR4. CCR4 is normally expressed on $CD4^+$ T_h2 cells and some other T-cell subsets, but is also abundant on most adult T-cell leukaemia lymphomas (ATLL) and cutaneous T-cell lymphomas (CTCL) [86, 90]. Mogamulizumab gained approval in 2012 for treatment of relapsed or refractory $CCR4^+$ ATLL, and later in 2014 for treatment of relapsed or refractory $CCR4^+$ CTCL [86]. Preclinical studies indicate that mogamulizumab acts only through ADCC or ADCP, as no CDC or direct cell death was observed [91]. Moreover, mogamulizumab engagement of CCR4 is claimed not to induce CCR4 internalisation, which might contribute to its favourable pharmacokinetics [92]. In addition, while various in vitro assays demonstrate the dominance of ADCC in mediating mogamulizumab efficacy, a non-glyco-engineered counterpart was not examined for comparison [91].

4.2 Obinutuzumab and the Anti-CD20 mAb Family

CD20 represents one of the most studied tumour targets with five of the 26 currently approved anti-cancer mAbs targeting this same receptor. The approval of rituximab in 1997 transformed the treatment of NHL and CLL [3, 93], and paved the way for subsequent antibody therapeutics. Although various CD20-targeting mAbs followed suit into the clinic, the precise mechanism of action of anti-CD20 antibodies in the clinical setting remains debatable, partly due to the large amount of incongruous data generated over the years by different groups [94]. In preclinical studies,

Table 1 List of fucose-engineered mAbs for cancer treatment

mAb name	Target	Modification	Technology	Condition	Phase	Results
Mogamulizumab	CCR4	Afucosylated	Potelligent	ATLL, CTCL	Approved	In relapsed $CCR4^+$ ATLL, ORR was 50%, 8 CRs, $n = 26$. Ref. [155] In CTCL, ORR was 39%, 13 PRs, $n = 38$. Ref. [156]
Obinutuzumab	CD20	Low fucose	GlycoMab	CLL, follicular lymphoma	Approved	In CLL, obinutuzumab + chlorambucil increased PFS to 26.7 vs. 16.3 months with rituximab + chlorambucil, $n = 781$. Ref. [87]. In rituximab-refractory indolent NHL, obinutuzumab plus bendamustine increased PFS to 22.5 months compared with 14.9 months with bendamustine monotherapy, $n = 396$. Ref. [95]
Trastuzumab[a]	Her2	Afucosylated	*FUT8* KO CHO	ND	Preclinical	ND
GA201	EGFR	Low fucose	GlycoMab	EGFR-positive solid tumours	Phase I	1 CR and 2 PRs in observed colorectal cancer patients, disease stabilisation observed in 27 patients, $n = 75$. Ref. [116]
KB004	EphA3	Afucosylated	ND	Advanced haematological malignancies	Phase I	3 AML patients achieved clearance of bone marrow blasts, 3 patients with myelodysplastic syndrome achieved SDs ranging from 2–12 months, OR observed in 4 patients, $n = 64$. Ref [118]
MDX-1342	CD19	Afucosylated	Potelligent	Relapsed or refractory CLL	Phase I	In CD19-positive relapsed or refractory CLL, 1 patient had PR, 6 had SD, 2 had DP, $n = 12$. Ref. [125]
MEDI-551	CD19	Afucosylated	Potelligent	Relapsed or refractory FL, DLBCL, CLL, MM	Phase I/II	In relapsed or refractory FL, DLBCL, CLL, MM, 3 patients experienced PRs and 2 CRs (lasted 9 months), $n = 25$. Ref. [129] In refractory CLL, 4 had PR and 13 had SD, $n = 20$. Ref. [128] In relapsed or refractory CLL, MEDI-551 + bendamustine demonstrated efficacy but results not further disclosed, $n = 147$. Ref. [157] In CLL, DLBCL, FL and MM, 9 had CR, 12 had PR, 42 had SD, median PFS about 9 months, $n = 83$. Ref. [158]
DI-B4	CD19	Low fucose	ND	CD19-positive indolent B-cell lymphoma	Phase I	Results pending
MDX-1401	CD30	Afucosylated	Potelligent	Refractory or relapsed HL	Phase I	In refractory or relapsed HL, 8 patients had SD (of which 2 had tumour burden reduction by at least 40%), 4 patients had DP, $n = 12$. Ref. [134]
SEA-CD40	CD40	Afucosylated	Metabolic interference[b]	Various advanced malignancies	Phase I	Patient recruitment in process
J6M0-mcMMAF	BCMA	Afucosylated	Potelligent	MM	Preclinical	ND

AML acute myeloid leukaemia, *ATLL* adult T-cell leukaemia lymphoma, *BCMA* B-cell maturation antigen, *CCR4* C-C motif chemokine receptor 4, *CHO* Chinese hamster ovary, *CLL* chronic lymphocytic leukaemia, *CR* complete response, *CTCL* cutaneous T-cell lymphoma, *DLBCL* diffuse large B-cell lymphoma, *DP* disease progression, *FL* follicular lymphoma, *HL* Hodgkin's lymphoma, *KO* knock out, *MM* multiple myeloma, *ND* not disclosed, *OR* objective response, *ORR* overall response rate, *PFS* progression-free survival, *PR* partial response, *SD* stable disease

[a] Afucosylated trastuzumab

[b] This production method is inferred from related patents filed by Seattle Genetics, Inc., the company which developed SEA-40

both Fc-mediated ADCC, ADCP, CDC and Fab-mediated direct cell death were evident for various anti-CD20 mAbs [94]. The current consensus indicates that the Fc domain, and in particular host activatory FcγRs, are required to recapitulate the clinical efficacy of anti-CD20 [12, 13]. The development of obinutuzumab, a low-fucose anti-CD20 mAb approved in 2013 for treatment of CLL [94] and in 2016 for rituximab-relapsed/refractory follicular lymphoma (FL) [95], was based on the premise that anti-CD20 mAb depletes malignant B cells through FcγRIIIa-mediated ADCC and/or ADCP [57, 96, 97]. Conceived from the GlycoMab platform, obinutuzumab was shown to mediate superior ADCC using human peripheral blood mononuclear cells as effectors to target CD20-expressing cell lines compared with the non-glyco-engineered obinutuzumab variant (i.e. with predominantly fucosylated glycans), and rituximab, an observation recapitulated in xenograft tumour models [97, 98]. Interestingly, the same study reported similar in vitro ADCP activity for all anti-CD20 mAbs examined regardless of the level of fucosylation [56, 98]. In addition, obinutuzumab has been reported to exhibit increased neutrophil activation and phagocytosis through the neutrophil restricted activatory FcγRIIIb compared with rituximab [56]. This is perhaps unsurprising as FcγRIIIb is highly homologous to FcγRIIIa. The two genes likely arose through a gene duplication event and, despite their similarity, differences in antibody binding affinity and cellular distribution may imply their differential immunological roles [99].

In 2014, data from a large phase III trial concluded that obinutuzumab in combination with chlorambucil gave more complete responses and improved progression-free and overall survival compared with rituximab plus chlorambucil in patients with untreated CLL and coexisting conditions [87, 100, 101]. Despite these conclusive clinical trial data, which many regard as a triumph for glyco-engineered anti-CD20 mAb therapy, questions remain over whether improvements conferred by obinutuzumab result from its low level of fucosylation, or from the fact that obinutuzumab is intrinsically a type II anti-CD20 mAb, a class of anti-CD20 antibodies associated with enhanced efficacy compared with type I anti-CD20 mAbs such as rituximab both in vitro and in multiple animal models [94, 102]. Type I and type II anti-CD20 mAbs differ in their mode of CD20 engagement and are typically characterised by their ability to redistribute cell surface CD20 molecules to lipid rafts, among other differences [97, 103, 104]. More importantly, type II anti-CD20 mAbs are much less prone to internalisation once bound to CD20 than type I mAbs, which are rapidly depleted from circulation [98]. This enhanced bioavailability of the type II obinutuzumab over the type I rituximab could contribute to the superiority of obinutuzumab in the trial, a hypothesis supported by a recent in vivo study demonstrating that the non-glyco-engineered, fucosylated form of obinutuzumab also outperforms rituximab in CLL clearance [105].

4.3 Trastuzumab

Trastuzumab, commonly known as Herceptin, targets the Her2 molecule and was originally approved in 1998 for treatment of Her2-positive early-stage breast cancer. Her2 is a cell surface receptor with tyrosine kinase activity, whose overexpression leads to constitutive signalling for cell survival [106]. Like rituximab, its precise mechanism of action remains unresolved. Although early reports suggested that trastuzumab acts by disrupting Her2 downstream signalling leading to apoptosis [107, 108], recent studies suggest the importance of ADCC/ADCP in its efficacy [109]. The clinical implication of the FcγR-mediated effector functions first emerged when one study found that patients treated with trastuzumab had better objective response rates when they possessed the high affinity V/V 158 FcγRIIIa allotype compared with patients with the low affinity F/F or mixed V/F allotypes [110]. Consistently, PMBCs isolated from patients with the high affinity V/V 158 allotype exhibited enhanced ADCC against Her2-positive human breast cancer cell lines [110]. On the other hand, genetic polymorphisms within the FcγRIIB or FcγRIIA were not found to influence therapeutic outcomes [110]. This apparent association of superior therapeutic efficacy with high affinity FcγRIIIa engagement warrants Fc-engineering to further improve trastuzumab activity. Indeed, an afucosylated version of trastuzumab was produced using the *FUT8* KO CHO cell line, which exhibits an 11-fold enhancement in ADCC against the Her2-amplified BT474-M1 cell line compared with its non-glyco-engineered parent [111]. Furthermore, afucosylated trastuzumab significantly improved the survival of tumour-xenografted animals transgenic for the human FcγRIIIa compared with the non-glyco-engineered trastuzumab, an effect lost when the hFcγRIIIa is absent, further demonstrating the superior efficacy of an afucosylated trastuzumab [111]. The enhanced ADCC observed in vitro and survival benefits in animal models warrant the continued clinical development of an afucosylated trastuzumab.

4.4 Afucosylated Anti-EGFR GA201

EGFR is a receptor tyrosine kinase whose expression and activation are upregulated in diverse epithelial cancers. The anti-EGFR mAbs, cetuximab and panitumumab, represent established treatments for colorectal cancer and head and neck cancer, respectively; however, the overall response rate and survival benefits remain modest, with patients

possessing KRAS mutations (of which a large proportion of these patients do) remaining unresponsive [112, 113]. Although anti-EGFR mAbs are purported to function through EGFR signalling blockade [114], it was hypothesised that their modest clinical efficacy might benefit from an engineered Fc. The low-fucose anti-EGFR mAb GA201 was therefore generated using the GlycoMab technology and shown to be approximately 85% afucosylated [115]. In vitro assays demonstrated that GA201 inhibits EGFR signal transduction to a similar extent as cetuximab; however, afucosylation boosted the ADCC activity of GA201 significantly above that of cetuximab or the non-glyco-engineered GA201 [115]. More importantly, GA201 exhibited ADCC activity towards tumour cell lines with KRAS mutations [115]. The in vitro efficacy was recapitulated in xenograft animal models in which GA201 treatment led to prolonged survival compared with cetuximab regardless of the tumour KRAS mutation status [115]. A phase I clinical trial also provided an early demonstration of GA201 activity in EGFR-positive solid tumours, including patients with KRAS mutations [116]. Interestingly, FcγRIIIa polymorphism status did not influence the activity of GA201 in these patients, in contrast to those involved in the trastuzumab trial discussed earlier [110, 116], potentially indicating that the glyco-engineering overcomes the lower activity associated with the low affinity allele, although this requires a further confirmatory study in larger cohorts to confirm.

4.5 Anti-EphA3

Similar to EGFR, EphA3 is a receptor tyrosine kinase belonging to the family of Eph receptor tyrosine kinases involved in the regulation of cell–cell communication and development [117]. Although there is no current anti-EphA3 mAb approved or indeed in late phase development, recently an afucosylated anti-EphA3 mAb, KB004, was reported to demonstrate modest efficacy in a phase I trial of relapsed and/or refractory haematological malignancies [118, 119]. The mechanism of action of KB004 remains unknown but presumably reflects those described above for EGFR mAbs.

4.6 Anti-CD19

CD19 is a ubiquitous marker expressed on B cells that associates with the B-cell receptor and acts to promote B-cell activation. Due to its widespread expression on B cells, including early progenitors, CD19 has been assessed as a target for numerous B-cell malignancies spanning acute lymphocytic leukaemia (ALL), CLL and NHL [120]. Despite ubiquitous antigen expression, the efficacy of the conventional anti-CD19 mAbs developed so far indicates inferiority to their anti-CD20 counterparts, with a notable lack of durable responses [121, 122]. Given their disappointing responses to date, antibody engineering has been attempted on these mAbs to boost activity. To date, the only approved CD19-targeting biological therapeutic remains a bispecific anti-CD19/CD3 comprising two scFv chains, which acts to recruit CD3-positive T cells to exert cytotoxicity against B cells [120]. In addition, multiple afucosylated anti-CD19 mAbs have been generated and are being clinically assessed. For example, MDX-1342, produced using the Potelligent technology, exhibits both enhanced ADCC and ADCP compared with its non-glyco-engineered version, and was able to mediate a higher level of B-cell depletion in non-human primates [123, 124]. A phase I trial of MDX-1342 to treat CD19-positive relapsed or refractory CLL demonstrated partial response in one of the nine patients recruited [125, 126]. MEDI-551 is another afucosylated anti-CD19 mAb, which, similar to MDX-1342, exhibits more potent ADCC in vitro than its non-glyco-engineered parent as well as efficacy in multiple xenograft animal models [124, 127]. In a phase I/II clinical trial in patients with relapsed or refractory FL, diffuse large B-cell lymphoma, CLL or multiple myeloma (MM), MEDI-551 demonstrated moderate activity as a monotherapy [128, 129]. A third afucosylated mAb, DI-B4, which has been transferred from Merck to Cancer Research UK as part of its Clinical Development Partnerships (CDP) programme, has recently been the subject of a phase I trial in CD19-positive indolent B-cell lymphoma. No clinical results have been released yet.

4.7 Anti-CD30

CD30 belongs to the TNFR family involved in the regulation of cell survival. Its expression in normal tissues is restricted to activated B cells and T cells but it is highly expressed on T-cell-derived tumours including anaplastic large-cell lymphoma and cutaneous T-cell lymphoma, making it a favourable tumour marker [130]. Currently, the only approved CD30-targeting antibody is brentuximab vedotin, an antibody–drug conjugate, whose efficacy stems from the cytotoxic agent monomethyl auristatin E [131]. One anti-CD30 mAb, MDX-60, demonstrated modest efficacy against Hodgkin's lymphoma (HL) and anaplastic large-cell lymphoma in a phase I/II trial [132]. Its afucosylated version, MDX-1401, was subsequently developed using the Potelligent technology and improved survival in a xenograft animal model [133]. Moreover, in a phase I trial involving twelve refractory or relapsed HL patients, MDX-1401 treatment led to a reduction in tumour burden for patients experiencing stable disease [134].

4.8 Anti-CD40

Like CD30, CD40 is a member of the TNFR family and has been extensively explored as a target for immunotherapy and anti-inflammatory applications [9, 135]. CD40 is ubiquitously expressed on various immune cells including B cells, macrophages, dendritic cells, as well as non-immune cells such as platelets and endothelial cells [136]. Although immunomodulatory (agonistic) anti-CD40 mAbs dominate the current focus for cancer research [9], some anti-CD40 mAbs were developed to target CD40-expressing B-cell lymphomas as direct-targeting mAbs aiming to block positive signalling from, or even mediate inhibitory signalling through, CD40; or to mediate Fc-dependent cellular destruction. Currently, only one anti-CD40 mAb, SEA-CD40, has been developed as an afucosylated therapeutic directed at CD40, which exhibits enhanced ADCC in vitro compared with its fucosylated parent SGN40 [137, 138]. The development of SEA-CD40, however, is at odds with the apparent mechanism of action of its parent antibody, SGN40. SGN40 demonstrates modest clinical activity in CLL and diffuse large B-cell lymphoma [139, 140], but it's in vivo efficacy, at least in one xenograft animal model, does not appear to require NK cell-mediated ADCC but rather depends on Fab-mediated apoptosis as full activity was demonstrated in mice lacking NK cells [141]. Consistently, the clinical activity of SGN40 demonstrated in a phase II trial indicates a lack of correlation between patient FcγRIIIa 158 V/F polymorphism and therapeutic activity [139]. Nevertheless, the enhanced engagement between an afucosylated mAb and the FcγRIIIa provides the potential to overcome the in vivo threshold for SGN40-mediated ADCC and offer synergy with Fab-mediated apoptosis.

4.9 Anti-B-Cell Maturation Antigen (BCMA)

The B-cell maturation antigen (*BCMA*) gene was originally identified from a translocation event in a malignant T-cell lymphoma patient and was later found to be preferentially expressed on mature B cells [142, 143]. It has since been established to be a member of the TNFR superfamily that interacts with B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) involved in B-cell survival [144, 145]. The ubiquitous expression of BCMA on MM cells, but not on other normal human cell populations and tissues, renders BCMA a direct target for MM [146, 147]. The first low-fucose anti-BCMA mAb J6M0, humanised from a murine parent and fucose-engineered using the Potelligent platform, had an additional functional feature whereby the cytotoxic drug monomethyl auristatin (MMA), a synthetic inhibitor of cell division, is conjugated to its Fc domain [147]. The Fc domain of such a low fucose antibody–drug conjugate (ADC) would be expected to not only induce enhanced cellular effector function via FcγRIIIA, but also exert direct toxicity towards target tumour cells. Consistent with this hypothesis, J6M0-MMA mediated efficient in vitro phagocytosis of MM cells by macrophages and reduced the MM cell viability independent of immune effectors [147]. Using immune-compromised animal models, the same group also reported superior in vivo efficacy of J6M0-MMA against human MM cell lines, compared with J6M0 alone [147]. Nevertheless, neither the murine parent nor a non-afucosylated variant of J6M0 were included in these experiments for comparison. While the MMA was covalently conjugated to the Fc protein backbone of J6M0, it is notable that with the coming of age of ADC, the unique chemistry of antibody Fc glycan (as opposed to the amino acid backbone) has been increasingly exploited as a site for cytotoxic drug conjugation [148–154]. The marriage between the intrinsic immune-modulatory properties of Fc glycan and its differential chemistry (from the protein backbone) could prove to be of great utility in the next-generation cancer therapeutics.

5 Conclusion

It is clear that we are now entering an era when non-canonical antibody therapeutics are becoming commonplace. With recent advances in glyco-engineering we possess the tools to fine-tune the ability of the Fc to elicit the desired interactions with potential receptors and interaction partners. The developments in afucosylation demonstrate a proof of principle that we can manipulate these interactions to elicit more efficacious responses from certain cellular effectors, potentially overcoming inter-patient variabilities in sub-optimal FcγR genotypes. However, the clinical experience illustrates that it is more important than ever that we fully understand the mechanisms of action of the parental mAbs in vivo before we undertake such studies—only then will the full potential of glyco-engineering be realised for patients.

Compliance with Ethical Standards

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