WHITE PAPER

Sugar with your protein? - Generation of a fucosylation-deficient CHO cell host to produce therapeutics with enhanced potency.

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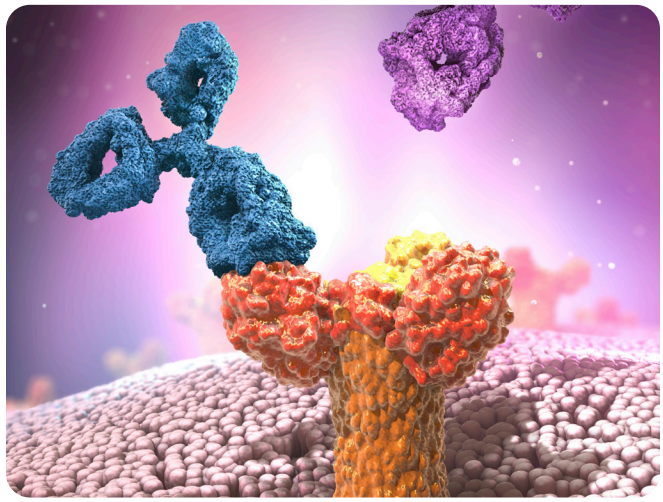
Monoclonal antibodies (mAbs) are highly specific, stable molecules with long half-lives and ability to interact and modulate different elements of the immune system[1]. Because of these properties mAbs have become a leading class of therapeutics in the treatment of a growing number of different disease areas from autoimmune diseases to neurological pathologies, infectious diseases, or cancer. Their versatility and the progress made in discovery and re-engineering of these molecules leading to improved therapeutic efficacy, have made them very successful with global revenue approaching 70% of total sales of biopharmaceuticals.

Increasing productivity whilst controlling product quality- A difficult balance

Early on, one of the main challenges in production of mAbs was low product titres. This was overcome by advances in cell line development, and new industrial bioproduction technologies that achieved better control over cells and substantial improvements in cell specific productivity[3]. Bioprocess optimisation, for example, has led to improvements in product titres and performance of the host cells. Generally, effort is put towards the optimization of fed-batch process with use of different media and feeds as well testing culture conditions (temperature, speed etc.)[4].



Now, the ever-increasing demand to develop and manufacture mAbs has led to biopharmaceutical companies investing heavily into research and development programs focusing on product quality and consistency. For biotherapeutics to be approved for use in human patients, a number of characteristics key to their performance and safety, termed critical quality attributes (CQAs)[5], need to be defined and controlled, for example presence and type of post translational modifications (PTMs) such as glycosylation[6].

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Glycosylation is an enzymatic process that involves the addition of oligosaccharide structures to specific amino acid sites of polypeptides, thus making them glycoproteins. This non-template based process occurs within the endoplasmic reticulum (ER) and Golgi[7] as the protein transits through the cell before secretion or translocation. There are many forms of glycosylation, but the two most common types are N-linked glycosylation and O-linked glycosylation:

- In N-linked glycosylation, the oligosaccharides are attached to the amide nitrogen of an asparagine (Asn) residue in a consensus sequence Asn-X-Ser/Thr where X is any amino acid except proline.
- In O-linked glycosylation, the glycans are attached to the oxygen atom of hydroxyl groups of amino acids such as serine (Ser), threonine (Thr) or tyrosine (Tyr)[8,9].

The glycan core structure (Figure 1) present on antibodies contains N-acetylglucosamine (GlcNAc) and mannose upon which other sugar residues such as galactose, sialic acid and fucose are added on.

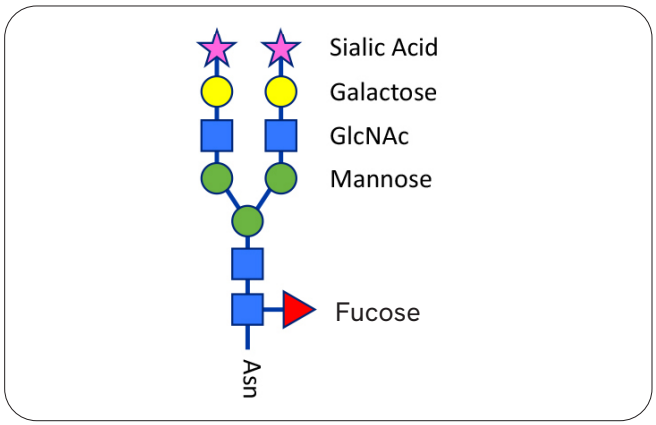

Figure 1: Glycan Core Structure.

Why is glycosylation so important in proteins?

Approximately 70% of mammalian proteins are glycoproteins with N-linked glycans, which often confer specific properties to the polypeptide chain. Abnormal glycosylation is known to be a causative factor in many human autoimmune diseases[10]. Also, variation in N-glycosylation of therapeutics can have a significant impact in protein folding, stability, pharmacokinetics, immunogenicity, or even mode of action[11]. This is particularly relevant in the case of monoclonal antibodies, where variability in the N-glycan structures present in the CH2 domain determines, amongst other things, cell-mediated responses such as Antibody-Dependent Cell Cytotoxicity (ADCC) and Complement Dependent Cytotoxicity (CDC). Specifically, the presence of a 1,6-linked fucose residue on the first GlcNAc of the core structure, is known to reduce drastically the ADCC activity elicited by antibodies[12,13,14,15].

Given the importance of the specific nature of glycosylation patterns in influencing the therapeutic effect of biotherapeutics, it is of no surprise that the increasing demand for biosimilars has made the control of the composition of the glycosylation patterns in biopharmaceuticals, a very important topic[16].

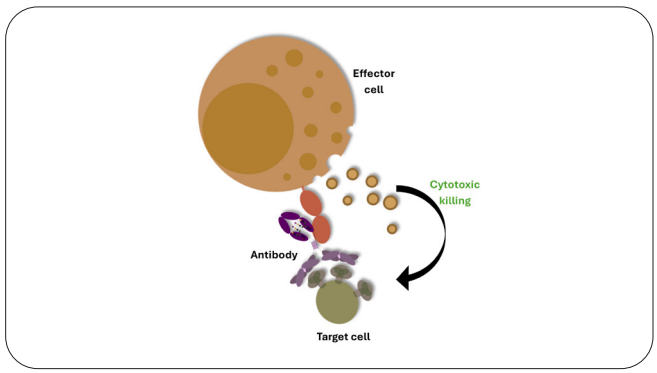

Figure 2: Antibody -dependent Cell Cytotoxicity (ADCC) mediated by NK cells: ADCC response on a cancer cell via CD16 receptor on NK cell, triggered with the help of a MAb (figure adapted[48]).

ADCC responses are mediated by the FcγRIIIa (CD16) receptor expressed primarily by natural killer (NK) cells, also called effector cells. Antibodies recognising specific ligands on a "target-cell" surface can activate NK cells through the interaction between the Fc region the antibody and the FcγRIIIa receptor expressed by NK cells, which in turn triggers the release of cytotoxic agents that ultimately eliminate the target cells[17]. It is known that the magnitude of ADCC response mounted by NK cells is dependent on the affinity between the FcγRIIIa receptor and antibodies[18]. Structural studies have revealed that the presence of fucose on the core glycan structure on IgG1 Fc leads to reduction in the binding affinity of the IgG1 to FcγRIIIa receptors in NK cells[13]. Therefore, the removal of core fucose in glycan structures of antibodies is an important strategy in the development of therapeutics for disease areas such as oncology where ADCC activity can be exploited as a suitable approach through which tumour cells can be removed[19]. This specific strategy is known as afucosylation, where the core fucose residue from the glycan core structure has been removed to produce monoclonal antibodies exhibiting higher affinities for FcγRIIIa receptors.

Advantages of non-fucosylated antibodies include [20]:

1. Ability to trigger ADCC responses against tumours exhibiting lower antigen expression levels[21]. This has been seen previously in Rituximab where it is clinically less effective against certain lymphomas with reduced CD20 expression levels [22]. The ability of afucosylated antibodies to elicit ADCC responses against cells with low antigen expression levels opens the door to more effective therapeutic approaches against so-far unsuitable oncology targets.

2. Reduced competition from serum IgGs in binding (and activating) FcγRIIIa receptors. It has been previously observed in clinical settings that the effectiveness of therapeutic antibodies such as trastuzumab can be inhibited by circulating IgG present in human serum, which compete for FcγRIIIa receptor binding [23]. To compensate and overcome this 'competition', higher levels of therapeutic antibodies are required, which can potentially introduce other complications and undesirable side-effects. Alternatively, the use of afucosylated antibodies can reduce such 'competition' from circulating serum IgG by increasing the binding affinity of a given therapeutic antibody to FcγRIIIa receptors.

By addressing these two factors, the use of afucosylated antibodies could have a significant impact in increasing the potency of biopharmaceuticals, expanding their therapeutic window and, by reducing the doses required to elicit a physiological effect, potentially reduce undesirable side-effects and complications associated to treatment.

The use of glycoengineered mAbs is not restricted to oncology therapies. For example, it is well-known that complement-dependent cytotoxicity (CDC) is affected by the glycosylation pattern present in antibodies, which can affect their binding to the Complement component 1q (C1q) complex. Antibodies exhibiting low or no galactose and high-mannose show a decreased binding to C1q leading to a reduced CDC response[24,25]. Further to this, it has been observed that highly sialylated antibodies can be used to mediate anti-inflammatory responses in autoimmune diseases[9].

Given the importance of glycosylation on effector functions mediated by therapeutic antibodies and Fc-fusion biotherapeutics, host cell lines used to express such products can be engineered to produce selective glycoforms that would in turn modulate their specific biological activity.

Right tool for the job

Chinese hamster ovary (CHO) cells have been long used as an expression system for production of biologics, since the approval of t-PA in 1987[26]. CHO cells can produce human-like PTMs and are robust systems that are able to adapt efficiently to different culture conditions, including their ability to grow in serum-free media. Also, importantly, CHO cells are less prone to being infected by human viruses. Recent advances in bioprocess engineering have dramatically increased the performance of these cells and the yields typically obtained in bioproduction[27,26].

CHO cells typically produce high proportion of mAbs containing fucosylated glycan core structure. This has an impact in the biological activity of antibody therapeutics expressed in CHO cells. Equally, as we have seen above, other glycan modifications can drastically influence the effector function of monoclonal antibodies. Therefore, there is great potential in the modification of the glycosylation pathways present in CHO cells to generate therapeutics with improved properties. For this purpose, the use of next-generation genome editing tools can offer an effective tool to generate engineered expression hosts able to produce therapeutics with specific characteristics[28].

Two glycoengineered mAbs lacking fucose have recently been approved for therapeutic use – anti-CCR4 Mogamulizumab and anti-CD20 Obinutuzumab[17] (both produced in genetically modified CHO cells). Many more glycoengineered monoclonal antibodies lacking fucosylation are currently in development in areas as diverse as oncology or infectious diseases such as Ebola[17].

Generating a new cell host to produce fully afucosylated therapeutics

For many years now, there has been a growing interest in developing methodologies to control the glycan composition in therapeutic proteins, particularly controlling fucose content in monoclonal antibodies as a strategy to generate more efficacious therapies. In order to enrich the proportion of afucosylated antibodies in the final product, several strategies have been explored: (1) control of cell host (CHO primarily) metabolism during cell culture conditions (for example through osmolality)[29], (2) the use of inhibitors targeting fucosyltransferase or other fucosylation enzymes[30], (3) expression of enzymes to deviate metabolism in order to reduce or deplete available fucose in the cells [30,31] , or (4) use of RNAi to repress or reduce transcription (and expression) of key fucosylation enzymes, amongst others.

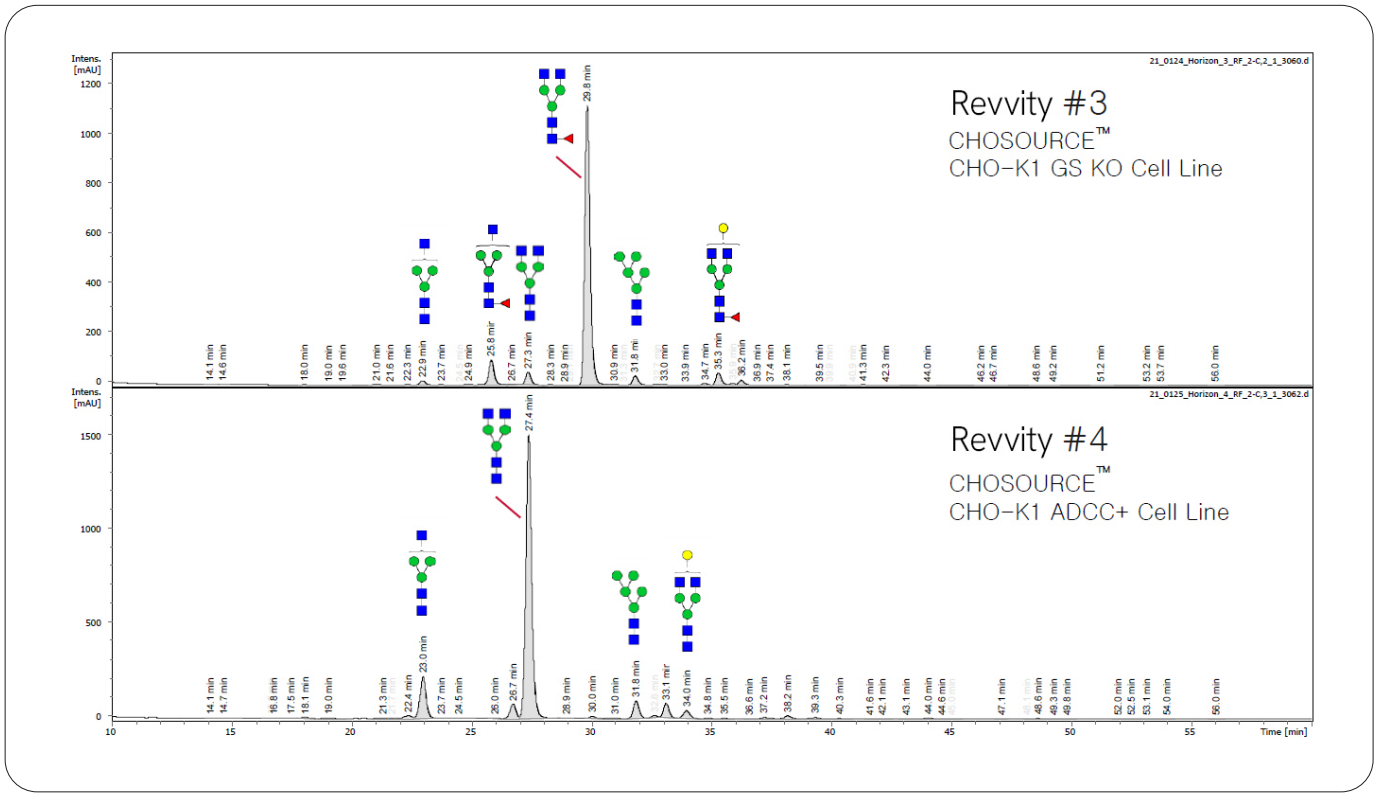

Figure 3: HILIC-UPLC analysis of released N-glycans: Glycan profiles of a trastuzumab (TTZ) control mAb produced in CHOSOURCE™ GS KO and CHOSOURCE™ ADCC+ cell line. TTZ produced in the ADCC+ cell line shows a shift towards the non-fucosylated glycan species as seen from the chromatogram.

One of the common challenges in bioproduction is controlling critical quality attributes (CQA) of the therapeutic products, particularly when linked to glycan composition. This is due to the fact that glycan composition is highly sensitive to process and media conditions, product and overall behaviour of cells in culture. This creates a problem for developers on two fronts: (1) the use of most of these technologies makes it virtually impossible to generate therapeutic preparations with 0% or 100% of their molecules containing a given glycan composition[30], and (2) the batch-to-batch variability observed in bioproduction that is intrinsically inherent to the nature of the cell culture control systems can have significant consequences in controlling drug potency and safety. The latter is particularly acute, since potency (and safety for that matter) cannot be just simply traced to 'dose' (i.e amount of antibody) anymore, given the batch-to-batch variations in glycan composition can have a substantial impact in drug potency (ADCC activity). This poses additional stresses on manufacturing and quality control, that are very difficult to address.

An alternative to such conundrum comes from the use of genetic host variants that lack entirely the ability to incorporate a fucose molecule in the glycan structure[32]. At Revvity, we built on our track record in gene editing and bioproduction solutions to develop a new CHO cell line for the production of afucosylated glycoproteins, the CHOSOURCE™ ADCC+ cell line. This new cell line has been generated from our CHOSOURCE™ GS KO cell line, which is a derivative from the original CHO-K1 clone[33]. The ADCC+ cell line has been obtained by generating a complete functional knockout of a fucosyltransferase gene, present in the parental CHOSOURCE™ GS KO cell line. The targeting strategy was based on the removal of one of the exons of the target gene, using Revvity's rAAV gene editing platform, to render an enzymatically inactive fucosyltransferase protein. Both alleles of the fucosyltransferase gene were targeted sequentially, where the second allele knockout was generated from one heterozygote clone containing a knockout of the first allele. On-target and off-target PCR screens were performed to validate the knocked-out alleles at each stage of the process[34,35].

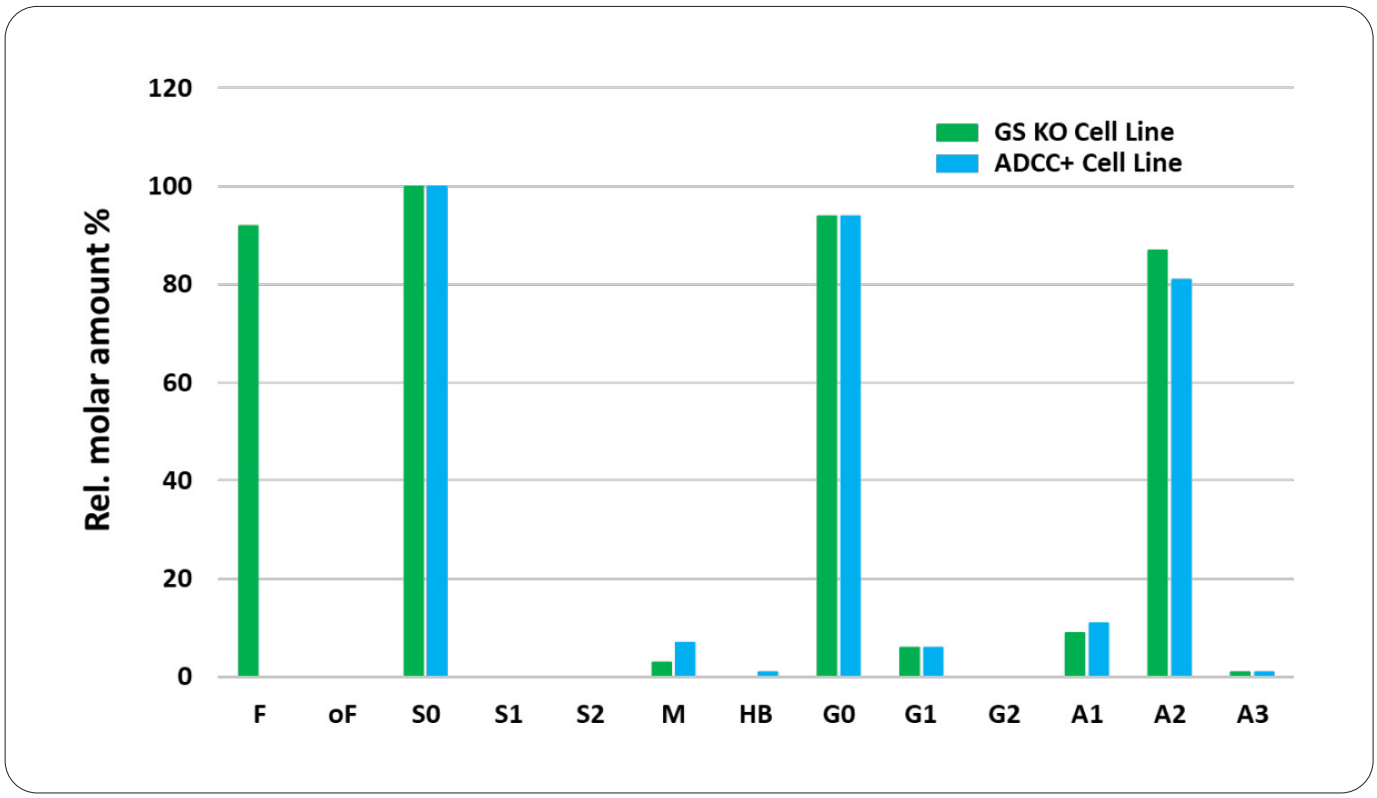

Figure 4: N-Glycan structures observed in model antibody expressed in GS KO and ADCC+ CHO cell lines. Complete absence of fucosylated species in trastuzumab (TTZ) expressed in CHO-K1 ADCC+ cell line compared to CHO-K1 GS KO cell line. F: core fucosylated; oF: N-glycans with outer-arm fucose; S0-S2: non- to bi-sialylated complex type; M: high mannose type; HB: hybrid type N-glycans; G0-G2: non- di-galactosylated complex type; A1-A3: mono- to tri-antennary complex type.

When a model antibody, in this case trastuzumab, is expressed in both the ADCC+ and GS KO cell lines, clear differences appear in their glycosylation profiles. The parental GS KO cell line-produced trastuzumab with intact core fucose, whereas the glycans on trastuzumab produced using the ADCC+ cell line are completely devoid of the core fucose, as it can be seen from the shift to the left in the chromatogram peak from fucosylated to afucosylated glycans (figure 3). Figure 4 further shows that that the percentage of fucosylated glycan has been reduced to 0% in the ADCC+ cell line whereas in the GS KO cell line it constitutes approximately 90% (figure 4).

To further validate the functionality of the new cell host in expressing antibodies lacking fucosylation, we proceeded to evaluating the biological activity of such afycosylated antibody variants (expressed in the ADCC+ cell line) compared to control, largely fucosylated, molecules (expressed in the parental GS KO cell line). For this purpose, we evaluated the ability of both types of molecules to elicit NK-mediated cytotoxicity against cells expressing different levels of HER2 antigen (the target recognised by our model antibody – trastuzumab). These 'target' cells used were T47D ($HER2^{Low}$) expressing low levels of the HER2 antigen, and SK-BR-3 ($HER2^{High}$) expressing high levels of the HER2. On the other hand, we wanted to assess the relative ability of the different variants to be recognised by two types of FcγRIIIa receptor containing either the F158 or V158 polymorphism. For many years it has been known that patients with Phe/Phe or Phe/Val genotypes show a reduced ADCC response to mAbs[36]. This creates difficulties when treating patients with different genetic make-ups and can potentially render some treatments ineffective.

The data in figure 5 shows how fully afucosylated antibodies expressed in the ADCC+ cell host exhibit a markedly higher efficacy in eliciting an ADCC response in all conditions tested, including target cells with low (T47D) levels of HER2 antigen expression and in the presence of NK cells expressing the F158 FcγRIIIa receptor polymorphism.

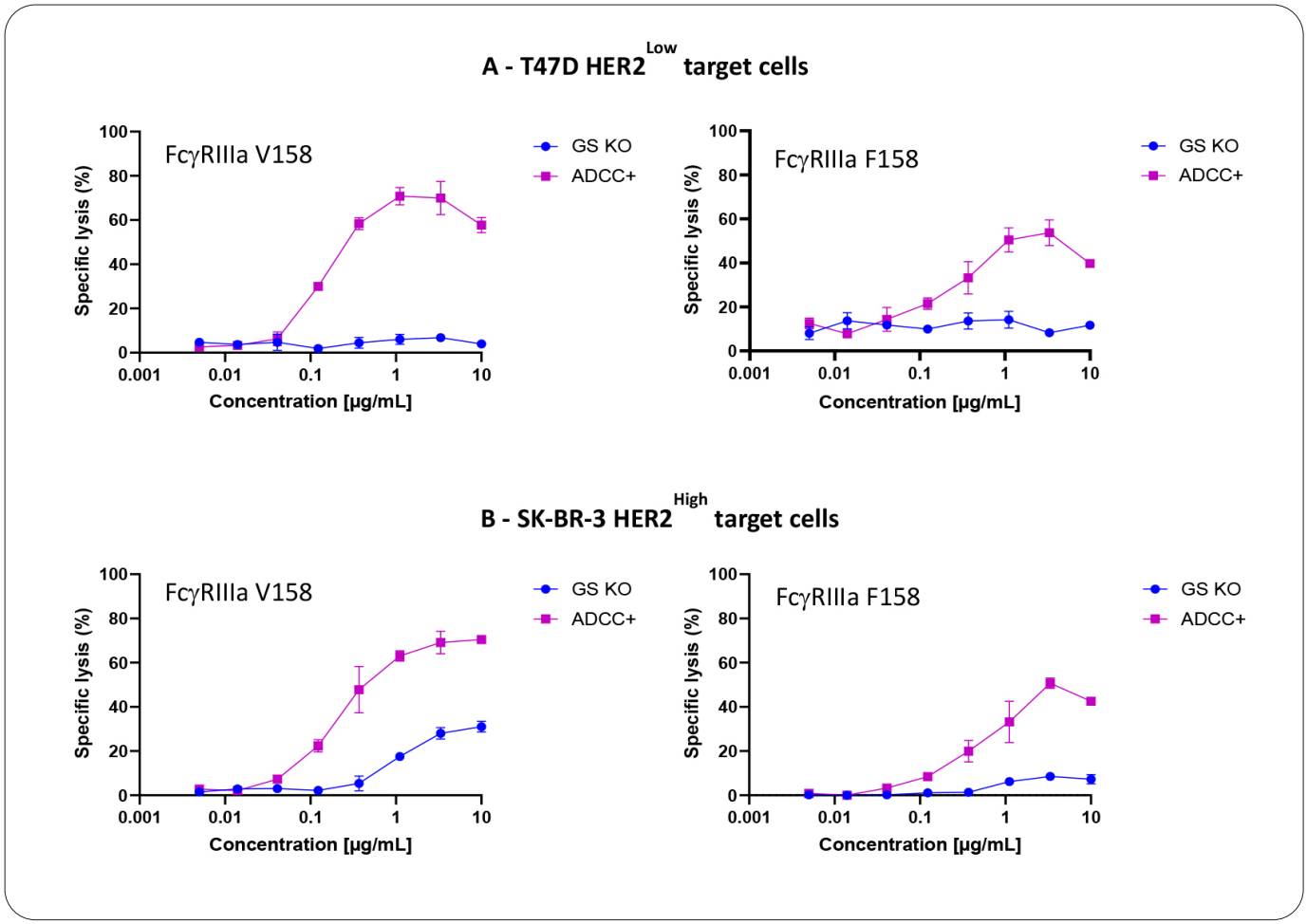

Figure 5: Effector function activity of afucosylated (ADCC+) and fucosylated (GS KO) trastuzumab. (A) T47D ($HER2^{Low}$) cells expressing low levels of HER2 antigen. (B) SK-BR-3 ($HER2^{High}$) cells expressing high levels of HER2 receptor. Fc γ RIIIa V158 correspond to effector NK cells expressing the Val polymorphism in residue 159, whereas Fc γ RIIIa F158 correspond to effector NK cells expressing the Phe polymorphism in the same residue. All panels show how the afucosylated antibody, expressed in the ADCC+ cell line shows substantially higher potency in eliciting an ADCC response.

Conclusion: Growing applications for glycoengineered antibodies with no fucose

As we have indicated above, glycan composition is well known to be able to modulate the biological activities of antibodies in our bodies, from regulating half-life, to control ADCC or CDC immune responses amongst others. Typically, these functions are mediated via endogenous Fc receptors present in different cell types and tissues and influenced by their relative affinity for different Fc architectures (including different amino-acid and sugar compositions). As we have mentioned, the composition of the glycan forming part of the Fc region of antibodies has become a focal point to modulate such functions.

Glycobiology is, therefore, emerging as an important discipline in the design of more effective biotherapeutics, particularly by modulating effector function in the case of IgG molecules. Gene editing technologies can effectively be used to engineer host cell lines able to produce afucosylated therapeutic antibodies, thus leading to enhanced ADCC response. It has been observed that antibodies that lack fucose in their Fc glycan shown an increase in their binding affinity to FcγIIIa receptors (up to 50-fold or more) in NK cells mediating effector ADCC responses [15,37]. The absence of this fucose residue also compensates for the differences in effector function activities across human populations with different polymorphisms in position 158 of the FcγIIIa receptor.

The use of afucosylated antibodies has shown to increase patient responses and outcomes, irrespective of the amino acid present at such position[15,37]. Moreover, this adaptive immune response has much wider applications beyond the development of treatments for oncology and opens the door to applications in a very wide range of conditions where better control over ADCC effector function activity is desirable. The development of antibodies with enhanced ADCC activity has been increasingly explored in the treatment of Infectious diseases, particularly viral infections. This has changed the paradigm that traditionally explored uniquely the neutralising aspect of therapeutic monoclonal antibodies for anti-infective applications. Indeed, there is a growing body of evidence supporting the use of cytotoxic mechanisms of action to control the spread of infection within patients affected by a given virus. This approach has been successfully assessed against a number of different viral infections, including Ebola virus[38, 39], Human Immunodeficiency Virus (HIV)[40], Respiratory Syncytial Virus (RSV)[41], or even influenza[42,15].

Interestingly, the use of effector function cytotoxic activity mediated by antibodies has also been explored in a very different category of diseases, including autoimmune indications. In these cases, elimination of undesired auto-immune responses is achieved by targeting or depleting B cells responsible for the abnormal auto-immune response[43,44]. For example, anti-CD20 (rituximab, ocrelizumab)[45,46] and anti-CD19 antibodies (Inebilizumab), are currently being used to treat some forms of multiple sclerosis and Neuromyelitis Optica Spectrum Disorder respectively[44,47] . Application of ADCC activity is also being explored to treat other auto-immune conditions[44].

We have shown how CHO cells can be genetically modified to enable production of afucosylated antibodies with enhanced ADCC activity, which can in turn make possible the development of more effective treatments in oncology, infectious diseases or autoimmune disorders. At Revvity we have developed a new host, our CHOSOURCE™ ADCC+ cell line, to support these types of application enabling better control over product quality and potency of new therapeutics.

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