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Glycans in immune recognition and response

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ABSTRACT

Glycans at the forefront of cells facilitate immune recognition processes. Cancer cells commonly present altered cell surface glycosylation, especially manifested in the expression of sialic acid at the termini of glycolipids and glycoproteins. Although tumor-associated carbohydrate antigens (TACAs) result in expression of *altered-self*, most such carbohydrates do not elicit strong humoral responses. Various strategies had been devised to elicit increased immunogenicity of such TACA aiming for potent immunotherapeutic antibodies or cancer vaccines. However some carbohydrates are immunogenic in humans and hold potential for novel glycotherapies. *N*-Glycolylneuraminic acid (Neu5Gc) is a foreign immunogenic sugar in humans originating from the diet (e.g., red meat) and subsequently expressed on the cell surface, especially accumulating on carcinoma. Consequently, the human immune system detects this *non-self* carbohydrate generating a broad anti-Neu5Gc antibody response. The co-existence of Neu5Gc/anti-Neu5Gc within humans spurs chronic inflammation mediated disease, including cancer. Concurrently, anti-Neu5Gc antibodies hold potential for novel targeted therapy. αGal is another foreign immunogenic carbohydrate antigen in humans and all humans have circulating anti-Gal antibodies. This review aims to describe the immunogenicity of Neu5Gc and its implications for human diseases, highlighting differences and similarities with αGal and its potential for novel targeted theranostics.

1. Introduction

The immune system discriminates *self* from *non-self* and eliminates particles carrying such non-self determinants. Pathogens can evade immune recognition either by masking non-self antigens and/or by disguising with host self-antigens through molecular mimicry. However, some pathological conditions present *altered-self* determinants that cause breaching of tolerance and lead to rejection through an autoimmune response.[103] Cell surface glycosylation is universal to all living cells and strategically positioned to mediate such immune recognition processes.[107,155]

Carbohydrate chains (glycans) that decorate glycoproteins and glycolipids (glycoconjugates) on the cell surface hold tremendous structural diversity.[120,155] In vertebrates, glycans usually terminate with sialic acids (Sia) that function as markers of normal self and can be recognized by a variety of receptors (e.g., Siglecs) mediating inter- and intra-cellular communication.[129,152] Cancer cells commonly present altered cell surface glycosylation as a result of abnormal expression of glycosyltransferases giving rise to changes in the typical glycan structures and/or to their expression levels.[11,153] Such changes especially affect sialylation patterns[33,85,86,122] that correlate with an advanced cancer stage, progression and/or metastasis.[11,57,78,122,139] Although such tumor-associated carbohydrate antigens (TACAs) result in expression of altered-self, most of these carbohydrates are largely poorly immunogenic and do not result in potent antibody responses,[60] with a few exceptions.[90,96] Nevertheless, carbohydrates have been shown to be involved in other aspects of tumor immunology and can contribute to cellular immune responses within immunoediting and immunosurveillance processes.[14,24,116,137] For example, it was shown that tumor cells with a lower degree of sialylation can interact better with immunosurveilling cells.[24]

TACA holds tremendous potential for targeted cancer therapy and in recent years efforts have been put into eliciting increased immunogenicity of such TACA aiming to generate potent antibodies for immunotherapy or a vaccine that can provoke a specific immune response against cancer.[52,71,164,166] Several approaches to improving TACA immunogenicity have been investigated: covalently coupling of carbohydrates to immunologically active protein carriers such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), tetanus toxoid (TT), or Bacille Calmette–Guérin (BCG);[52,166] treating with a mixture of several mono-epitopic vaccines;[95,133] or fully synthetic homogeneous vaccines designed to contain an adjuvant or other immunological epitopes to further enhance the immunogenicity of resulting vaccines.[12,13,73] Many of these new strategies were very successful at improving

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immunogenicity, at least in animals.[36,52] However, although several promising TACA-based cancer vaccines have entered clinical trials (including in Phase III),[34,77,100] none have been approved for clinical use yet. Most tested vaccines failed in clinical trials mainly due to the lack of a robust T cell-mediated immunity and/or lack of survival benefit for patients.[34,36,52,77,94,100,166,172] Importantly, many of the new strategies that trigger much stronger immune responses have not yet been tested in humans.[26,71,164]

αGal is a foreign immunogenic carbohydrate antigen in humans due to a specific gene inactivation, and all humans have circulating anti-Gal antibodies that could potentially be used for immunotherapy if the antigen had been present on the target cells.[42] αGal was used to generate autologous tumor-vaccines by incorporation of this xenogenic carbohydrate antigen through intra-tumoral injection of αGal glycolipids.[47] This proved to then tag the cells for destruction by complement-mediated cytotoxicity (CDC) and by antibody-dependent cellular cytotoxicity (ADCC) following anti-Gal binding to the αGal epitopes *de novo* expressed on the tumor cells in mice.[42,43,47] Another potent approach to increase tumor immunogenicity was to metabolically engineer cells to express unnatural TACA analogues[18] followed by treatment with antibodies specifically generated against these unnatural carbohydrates that could promote CDC *in vitro*, though evidence for therapeutic efficacy *in vivo* is still pending.[52,159]

However, recent research suggests that such metabolic engineering with a foreign carbohydrate actually occurs in humans through dietary consumption of *N*-Glycolylneuraminic acid (Neu5Gc) that accumulates on carcinoma and also provokes an immune response in humans.[99,115,122,123,127,146,150,174] Neu5Gc is a non-human sialic acid since humans uniquely cannot synthesize it due to a specific inactivation of the gene encoding the enzyme CMP-Neu5Ac hydroxylase (*CMAH*).[22,151] Thus both αGal and Neu5Gc are immunogenic in humans because they are foreign to the human immune system, and therefore serve as targets for circulating antibodies.[98,126,141] This review aims to summarize recent research on the immunogenicity of Neu5Gc and its implications for cancer and other human diseases, emphasizing its potential for novel targeted theranostics (therapy and diagnostics). In addition, the differences and similarities between the immunogenic sugars αGal and Neu5Gc will be highlighted.

2. Sialic acid diversity

Sialic acids (Sias) are a diverse family of ~50 alpha-keto aldonic acid carbohydrates with a nine-carbon carboxylated backbone, found predominantly as the terminal units on glycans and glycoconjugates in vertebrates.[4] Sia diversity arise from various modifications at either the C5-amino group (with acetyl or glycolyl) or the hydroxyl groups at C4, C7, C8, and C9 by acetate, lactate, sulfate, or phosphate esters or by methyl ethers.[4,140,154] The two most common Sias in mammals are *N*-Acetylneuraminic acid (Neu5Ac) and its hydroxylated form *N*-Glycolylneuraminic acid (Neu5Gc), as described in Figure 1[140,154]. Sia is α-linked to underlying sugars through their C2 to either C3/6 of galactose, C6 of *N*-acetylgalactosamine, or to C8 of another Sia (α2-3Gal or α2-6Gal; α2-6GalNAc; and α2-8Sia, respectively).[4] Overall sialoglyconjugate diversity results from Sia-modification, linkage to underlying sugars and their composition, the conjugated scaffold (protein/lipid), the glycan mode of attachment (e.g., *O*/*N*-linked to proteins), and finally their spatial organization (density).[4,25,140]

3. Recent advances in the synthesis of sialic acid derivatives

Chemical sialylation was one of the most challenging glycosylation reactions in the past largely due to low yields, poor stereo-selectivity, and difficulties in product purification.[19] Recent advancements in chemical and especially chemoenzymatic synthesis provided chemically well-defined and structurally homogeneous sialic acid-containing glycans (sialosides).[1,10,84,112] The newly developed chemical 'one-pot glycosylation' reaction allowed direct sequential assembly of monosaccharides into glycans in the same reaction flask without intermediates isolation,[70,158] however this approach remains impractical in generating sialosides, especially for certain Sia-derivatives that are labile to the final deprotection steps.[112] Importantly, subsequent development of a highly efficient one-pot multiple-enzyme (OPME) system allowed the chemoenzymatic synthesis of naturally occurring and non-natural sialosides.[1,15,112,167,170,171] This approach exploits the high regio-selectivity, chemo-selectivity, and stereo-selectivity of enzymes with the flexibility and diversity of chemical synthesis to achieve an efficient synthesis of such complex carbohydrates.[1,112] This breakthrough allowed to generate a wide collection of sialosides[15,19,20,32,93,142,167–171] finally paving the way for extensive investigation of sialic acid's diversity and their biological roles.[19,21,29,83,124,143]

4. Hanganutziu–Deicher (HD) antigens and antibodies

Almost a century ago human 'heterophile' antibodies had been noticed and later on suggested to recognize Neu5Gc-antigens. In the 1920s Hanganutziu[54] and Deicher[28] independently noticed that injection of horse antisera (e.g., to tetanus toxin or diphtheria) into humans caused 'Serum-sickness' with allergy-like symptoms due to hemagglutinins. These human 'heterophile' antibodies later named Hanganutziu–Deicher-(HD)-antibodies could agglutinate animal erythrocytes from many species, except human and chicken. Such antibodies were then detected in patients who had never been exposed to animal sera, including patients with various inflammatory or infectious conditions and cancer (reviewed in[99]). Subsequently HD-antigens were defined in late 1970s as a Neu5Gc-containing ganglioside (Neu5Gc-GM3, Neu5Gcα2-3Galβ1-4G1cβ1-1'Ceramide)[5,64,104] or Neu5Gc-containing glycoprotein.[111,118] However, these early studies used crude methods for detection of the HD-antigens[39,62,65,69,72,80,81,87,119,138] or HD-antibodies,[108,109,114,117,145] apparently assuming that normal humans are negative.[53,75,114] With the advent of modern glycobiology tools detection of Neu5Gc-antigens and anti-Neu5Gc antibodies had been extensively revisited providing compelling evidence for their presence not only in patients but also in healthy individuals, followed by investigation of their implications for various human diseases, as described below.

5. Neu5Gc in human tissues

5.1. Neu5Gc in healthy humans

Healthy human tissues had been inspected in the past by various chromatographic and immunochemical techniques for detection of Neu5Gc, however those failed to provide unequivocal chemical evidence for its presence[99] or failed to detect it at all.[82] The expression of this sialic acid had been recently re-examined mainly by highly characterized antibodies to Neu5Gc-containing antigens, HPLC, and finally mass-spectrometry (Table 1). Varki and colleagues had generated a highly sensitive polyclonal chicken anti-Neu5Gc antibody (chickens immunized with GM3(Neu5Gc)) that was further affinity-purified and extensively characterized by various protocols (e.g. ELISA, Western blot, flow cytometry, immunohistochemistry, and glycan microarray)[31,124,146] demonstrating broad monospecificity to various Neu5Gc-containing glycoconjugates.[124] Immunohistochemistry staining with this

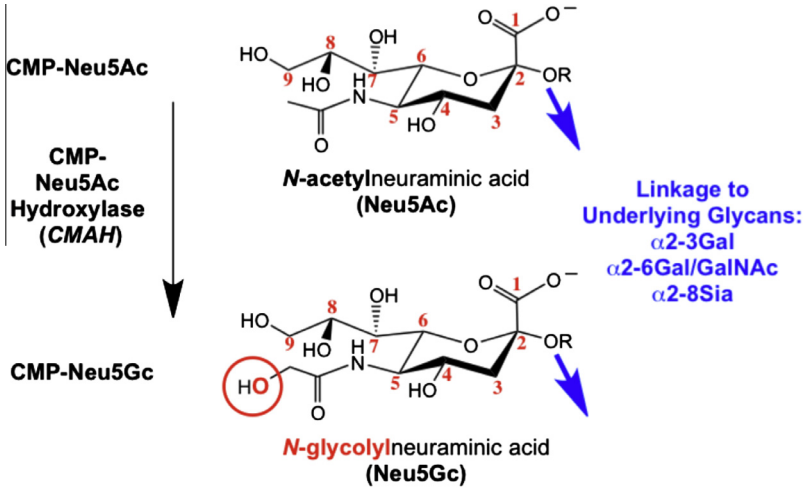

Figure 1. Schematic diagram of Neu5Ac and Neu5Gc that differ by a single oxygen atom.

Table 1
Detection of Neu5Gc in normal and malignant human tissues

Human Tissue	Normal	Cancer
Brain		
Breast	A[16,31]	A[16,66,102,144,146], H[58], MS[102]
Colon/colorectal	A[31,146]	A[62,66]
Fetal stomach	A[146]	
Gastric		MS[82]
Heart	H[110,146], MS[146]	
Kidney	A[9,31,146]	
Intestine	A[16,31]	
Liver	A[146], H[110,146], MS[146]	MS[82]
Lung	A[146]	A[56,149]
Ovary	A[9]	A[31], H[58]
Pancreas	A[146]	H[58]
Placenta	A[146]	
Prostate	A[31,146]	
Skin/melanoma	A[146]	A[16,31,68,81,138]
Spleen	A[31,146], H[110,146], MS[146]	
Testis	A[146], H[110]	
Uterus	A[146]	A[66], MS[30]
Chondrosarcoma		A[63]
Germ cell tumors		A[105]
Leukemia		A[66]
Malignant lymphoma		A[66], MS[82]
Nasopharyngeal		A[66]
Neuroblastoma		A[31]
Retinoblastoma		A[67]
Teratoma		MS[82]

Most reports used chicken antibodies (A) recognizing Neu5Gc-containing antigens for immunohistochemistry or immune-staining of TLC, while only few used HPLC (H) or mass spectrometry (MS).

chicken anti-Neu5Gc IgY established for the first time the presence of low levels of Neu5Gc mostly in epithelium and endothelium in normal human tissues.[31,146] Neu5Gc was detected on blood vessels endothelium and epithelium or secretory epithelia cells of lung, skin, colon, prostate, uterus, kidney, spleen, testis, pancreas, liver, and fetal stomach.[31,146] In addition, normal human placenta and most normal human tissues (either frozen or paraffin sections) consistently showed staining of blood vessels endothelium, and sometimes also of the glandular epithelial cells of breast, luminal edge of colonic mucosal epithelial cells, crypt epithelium of small intestine, some glandular epithelium of prostate, kidney glomeruli and interstitial capillaries, and lung bronchial epithelium.[31] Another well-characterized antibody is the 14F7 murine monoclonal IgG_1 antibody generated by immunizing mice with GM3(Neu5Gc) hydrophobically conjugated with very low-density lipoproteins (VLDL).[16] This antibody was shown to be highly specific for the ganglioside GM3(Neu5Gc) without cross-reactivity with GM2(Neu5Gc) or their Neu5Ac-counterparts or a sulfated glycolipid.[16,135] This antibody stained kidney and ovary normal tissue samples, but did not stain testis, prostate, and bladder.[9] However, in addition to membrane staining, this antibody could also strongly stain the cytoplasmic region of breast malignant tumor cells suggesting it may also recognize other antigens, likely Neu5Gc-containing glycoproteins.[16] It is worth noting that 14F7 had largely been tested in the presence of animal products (i.e. BSA)[16] that are likely contaminated with Neu5Gc-antigens and therefore may reduce its efficacy.[126] Nevertheless, specificity of anti-carbohydrate antibodies may be ambiguous,[101] therefore conclusive evidence for the presence of Neu5Gc finally came from other detection methods. A highly sensitive HPLC was able to detect Neu5Gc at 1–3% of the total Neu5Ac in human liver, spleen, heart, and testis,[110] and this was later also confirmed by mass spectrometry analysis of glycopeptides and some glycolipid fractions from human kidney, heart, liver, and spleen.[146]

5.2. Neu5Gc in cancer patients

In contrast to the limited evidence for the presence of Neu5Gc in normal human tissues, its presence on various cancers had been widely described (reviewed in Ref. 99; Table 1). Neu5Gc was detected by immunofluorescence on breast, colorectal, nasopharyngeal, uterine, leukemia, and malignant lymphoma,[66] as well as in ovary, pancreas, embryonal, adenoidocystic, and teratoma.[66] By thin-layer chromatography (TLC) immunostaining, Neu5Gc was detected in colon[62,69,106] melanoma[68,81] retinoblastoma,[67] yolk sac tumor,[105] and breast carcinoma.[102] When using immunohistochemical methods, Neu5Gc was detected in colon,[160] embryonal carcinoma, teratocarcinoma, choriocarcinoma, yolk sac tumor,[105] melanoma,[16,138] and breast.[16,144] In addition, chemical analysis of cancer samples showed Neu5Gc in chondrosarcoma,[63] gastric, liver, malignant lymphoma, teratoma,[82] breast,[55,102] and uterine.[30] More recently, the highly specific polyclonal chicken anti-Neu5Gc IgY[31,124,146] was shown to detect Neu5Gc in most breast tumor cells and related blood vessels,[146] as well as in melanoma and neuroblastoma.[31] Moreover, malignant ovarian carcinoma showed staining of the tumor itself together with their angiogenic blood vessels.[31] Neu5Gc was also detected by histochemical staining of non-small cell lung cancer with 14F7 monoclonal antibody.[149] Using HPLC, Neu5Gc was detected in ovarian, breast, and pancreatic cancers at 1–4% of the total sialic acids, which is a higher

percentage than what was found for normal human tissues.[110] Finally, Neu5Gc in malignant tissues was also confirmed by mass-spectrometry.[30,82,102]

5.3. Metabolism of Neu5Gc in humans

Humans lost the ability to synthesize Neu5Gc in light of an irreversible exon deletion in the *CMAH* gene encoding the CMP-Neu5Ac hydroxylase (Fig. 1).[23,74] Similarly, *Cmah*$^{-/-}$ knockout mice cannot synthesize Neu5Gc,[59,113] suggesting that the source for Neu5Gc in human tissues is exogenic. *CMAH* loss is unique to humans in contrast to all other mammals therefore consumption of red meat and other mammalian-derived food products (Fig. 2) likely replenishes Neu5Gc in human tissues.[146] Uptake of Neu5Gc into cells *in vitro* occurs primarily by macropinocytosis into endosomes with subsequent delivery into the cytosol via a lysosomal transporter, then it is further activated in the nucleus and finally incorporated into cell surface molecules in the Golgi as if it was originally made in the same cell.[7] This process is further enhanced by high cell growth rates.[115] It was also demonstrated that dietary Neu5Gc-containing glycoproteins, but not free Neu5Gc, can metabolically incorporate into *Cmah*$^{-/-}$ mice *in vivo* in a human-like tissue distribution.[6] Finally, the metabolic Neu5Gc-degrading pathway in mammalian cells has recently been elucidated.[8]

6. Circulating anti-Neu5Gc antibodies in humans

6.1. Anti-Neu5Gc antibodies in healthy individuals

The advent of modern chemoenzymatic synthesis of various sialoglycoconugates allowed revisiting claims of lack of anti-HD antibodies in healthy humans.[53,75,114] Using chemically defined sialoglycans it was shown that all normal humans have circulating anti-Neu5Gc antibodies.[115,127,146,174] Furthermore, the extent of response seemed to reflect the diversity and complexity of sialoglycans revealing a broad and variable spectrum of anti-Neu5Gc antibodies (Fig. 3).[127] These anti-Neu5Gc antibodies constituted IgM, IgA, and most commonly IgG[115,127] that ranged at ~0.1–0.2% of total Igs (ranging at 0.1–23 μg/mL) against several potential targets.[127] These antibodies could be affinity-purified from individual human sera or from pooled human IgG and showed that they could specifically bind to human carcinomas that had accumulated Neu5Gc *in vivo*.[97,127] Furthermore, it was demonstrated that anti-Neu5Gc IgM and IgG arise in infants at 6 months, soon after the introduction of Neu5Gc in the diet (e.g., cow's milk formula and baby foods containing red meat), and are likely induced via dietary-Neu5Gc uptake by commensal bacteria.[148] This response can further be enhanced in certain pathological conditions[125,141] and can sometimes remain high for over 30 years.[141] Hence, like a 'Trojan horse', Neu5Gc metabolically incorporates into human cells as '*self*' but then becomes presented on the cell surface in the context of novel '*non-self*' antigens thus termed 'xeno-auto-antigens' and 'xeno-autoantibodies'.[127]

	Food item	g/d	Neu5Gc daily intake (μg)
Red Meat	Beef	250	7525
	Pork		6375
	Lamb		4550
Milk Products	Goat cheese	120	4788
	Cow milk (raw)		943.2
	Cow milk (2%)		928.8
	Cow cheese		768
	Butter	50	60
Chicken/Fish	Salmon	250	367.5
	Chicken		19
	Turkey		11.5
	Cod		10
	Tuna		8
	Duck		5

Figure 2. Summary of Neu5Gc content in various food items according to gram per day consumption (g/d) (modified with permission from Ref. 146; Copyright (2003) National Academy of Sciences, U.S.A.).

6.2. Implications of anti-Neu5Gc antibodies in cancer

6.2.1. Low dose anti-Neu5Gc antibodies in tumor progression

Several epidemiological studies suggested a connection between certain dietary foods (e.g. red meat) with an increased risk of chronic diseases including cancer.[128,136,147,163,173] Thus the co-existence of Neu5Gc with circulating anti-Neu5Gc *in vivo* prompted investigation of their contribution to pathological outcomes, especially in light of the numerous reports on their detection in cancer.[99,174] The effect of anti-Neu5Gc antibodies on tumor growth was tested in the Neu5Gc-deficient (*Cmah*$^{-/-}$) mouse model. When these mice were injected with Neu5Gc-positive tumors and then treated with low dose anti-Neu5Gc antibodies (either anti-Neu5Gc mouse serum or affinity-purified human anti-Neu5Gc IgG) the resulting tumors grew larger compared to the control treated mice.[58] The enhanced tumor growth was suppressed by an anti-inflammatory treatment suggesting the effects were mediated by chronic inflammation and this notion was further supported by the detection of infiltration of inflammatory cells.[58] This had suggested that the co-existence of Neu5Gc with circulating anti-Neu5Gc antibodies might serve as the missing molecular link between diet and cancer risk.[128] Likewise, dietary-Neu5Gc was shown to incorporate into human endothelium whereby anti-Neu5Gc antibodies could induce complement deposition and endothelial activation, suggesting they can initiate, propagate, and/or exacerbate an inflammatory response at the endothelium, potentially playing a role in vascular inflammation disease states such as atherosclerosis.[130] Together, these findings implied a novel disease concept in which chronic inflammation-mediated disease is induced by a metabolized dietary-sugar that also stimulates an immune response.[58] It was also later supported by follow up studies in humans facilitated by the development of a specialized sialoglycan-microarray with multiple Neu5Gc-/Neu5Ac-glycans.[123] This array allowed high-throughput screening of anti-Neu5Gc IgG responses in sera samples of cancer versus non-cancer patients and led to discovery of the novel antibody carcinoma biomarker, anti-Neu5Gc-Sialyl-Tn IgG.[123] Neu5Gc-Sialyl-Tn resembles the well-known cancer-associated carbohydrate antigen Sialyl-Tn (STn), only that Neu5Gc replaces Neu5Ac at the terminal postion.[123] Further longitudinal population studies are required to establish anti-Neu5Gc antibodies as potential biomarkers for increased risk of cancer and other chronic inflammation mediated diseases. Recently, a novel method for detection of the total polyclonal anti-Neu5Gc antibodies response was developed and may facilitate high-throughput screening of human sera samples for such high-throughput studies.[125]

6.2.2. High dose anti-Neu5Gc in tumor regression

Incorporation of Neu5Gc into cancer cells results in modification of the cell surface glycans resulting in a representation of an *altered-self* pattern. Anti-Neu5Gc antibodies could potentially recognize these unique neo-markers on cancer cells and therefore

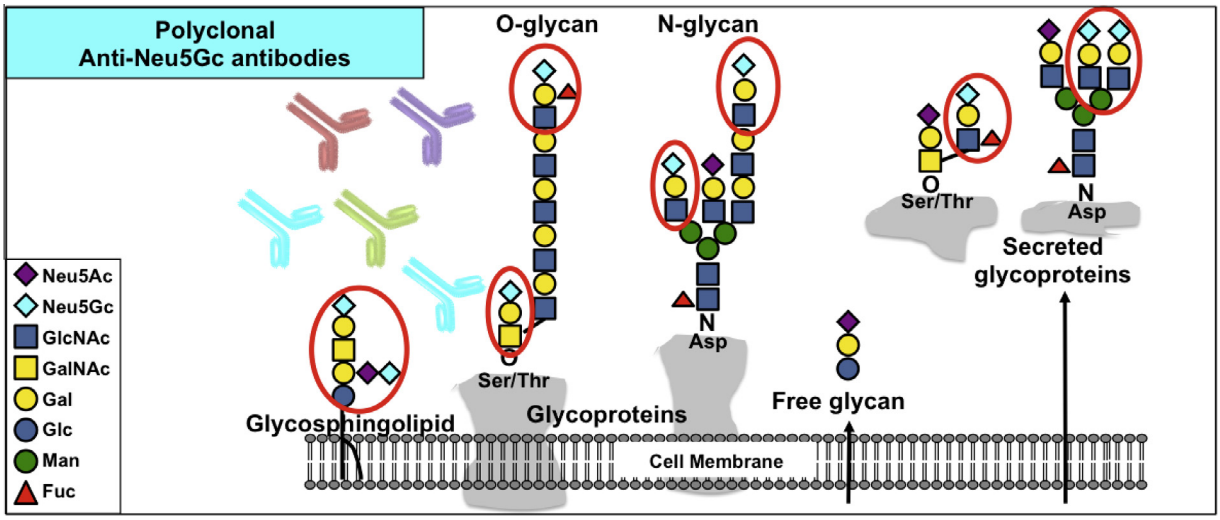

Figure 3. Schematic diagram of the diversity of anti-Neu5Gc response to multiple Neu5Gc-containing antigens (circled in red) on various cell surface glycans. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

could theoretically be used for immunotherapy. In line with that, it was shown that human sera with high anti-Neu5Gc humoral response could promote complement-dependent killing of leukemic cells.[115] Likewise, human serum or affinity-purified IgG with high reactivity to Neu5Gc-Sialyl-Tn was able to promote killing of human cancer cells expressing this unique antigen by both complement- or antibody-dependent cellular cytotoxicity (CDC or ADCC).[125] In addition, further *in vivo* studies in the *Cmah*$^{-/-}$ mice model demonstrated that a high dose of affinity-purified human anti-Neu5Gc IgG inhibited tumor growth.[123] Altogether, the results suggested that these antibodies' impact on the immune response may be dependent on their dose: while a low dose of affinity-purified human IgG supported tumor growth,[58] a higher dose inhibited tumor growth.[123]

7. Clinical potential of anti-Neu5Gc antibodies

Neu5Gc-glycoconjugates have been investigated as potential cancer vaccines, especially targeting gangliosides (sialylated glycolipids).[34,35,37,61] Certain gangliosides (e.g. GM2 and GM3) are over expressed in cancer.[38,56,89,102,165] These gangliosides can metabolically replace Neu5Ac with Neu5Gc and it was reported that GM2(Neu5Gc) ganglioside is found at high levels in several cancers and can be further increased by hypoxia-induced transcription of a sialic acid transporter.[79] Similarly, GM3(Neu5Gc) was described as a cancer-associated antigen and potential therapeutic target and cancer vaccine.[34,35,56,91] Currently there are two clinical trials targeting GM3(Neu5Gc): (i) The antibody 1E10,[3] commercially known as Racotumomab, is a monoclonal antibody but is used as a cancer vaccine. This is a murine monoclonal antibody (IgG_1,κ), which was obtained from BALB/c mice immunized with the purified P3 antibody (murine monoclonal IgM, κ, that reacts specifically with a wide range of Neu5Gc-gangliosides, sulfated glycolipids, and with antigens expressed in human breast tumors[156]) coupled to KLH (Keyhole Limpet Haemocyanin) in Freund's adjuvant.[157] Thus, Racotumomab is an anti-idiotypic antibody reflecting a mirror image of the P3 antibody and is currently being tested in a randomized, controlled Phase II/III clinical trial.[51,132,134] There is compelling evidence that Racotumomab can elicit a strong humoral and cellular immune response that has a positive impact on patient's survival.[2,51] However, since the P3 antibody is of broad specificity it is possible that Racotumomab may not be strictly reflecting GM3(Neu5Gc). (ii) The alternative vaccine is liposome-based, in which GM3(Neu5Gc) was incorporated into very small-sized proteoliposomes (VSSP) derived from *Neisseria meningitides* (also known as NeuGcGM3/VSSP). Patients treated with this vaccine showed benefit in progression free survival and overall survival.[17,27,121,132] This vaccine seems to be safe with presumable specific humoral and cellular immune responses in patients and is currently being further investigated in Phase III clinical trials.[132]

Another clinical aspect of anti-Neu5Gc antibodies involves glycosylation of biotherapeutics. Neu5Gc can be presented on clinically-used biotherapeutic glycoproteins (e.g., antibodies, inhibitors, cytokines etc.), likely due to usage of non-human mammalian cell lines and/or the addition of animal-derived tissue culture supplements during their production.[49,50] In a Neu5Gc-deficient mouse model (*Cmah*$^{-/-}$), it was shown that when Neu5Gc-bioterapeutics is given to mice who have circulating anti-Neu5Gc antibodies, immune complexes are generated mediating clearance of the drug and resulting in reduction of its effective concentration and efficacy.[49] This is likely also relevant in humans and may possibly explain common variability in efficacy of glycosylated-biotherapeutics. Consequently, if a patient that has circulating anti-Neu5Gc antibodies is treated with Neu5Gc-biotherapeutic the drug's efficacy might decrease. Similarly, anti-Neu5Gc antibodies may be involved in rejection of human cells prepared for allo-transplantation and auto-transplantation if those were grown in animal-derived tissue culture supplements.[50,126] Finally, these effects are likely to vary between individuals reflecting the extent of the anti-Neu5Gc response as demonstrated by the antibodies level, recognition pattern, specificity, and isotype. Therefore, screening of patients for anti-Neu5Gc response should be considered as a variable for personalized-therapy.[122] Recently, a simple method to screen for the overall anti-Neu5Gc response had been developed and may facilitate patient evaluation prior to selection of therapy.[125]

8. Immunogenic alpha-Gal (αGal) xenoantigen in humans

In addition to Neu5Gc, another immunogenic sugar antigen in humans is the αGal that had been thoroughly investigated by Galili and colleagues.[41,42] αGal shares some common features with Neu5Gc however there are some major differences in other critical aspects as highlighted in Table 2. While Neu5Gc can be found conjugated to multiple glycans to generate a collection of antigens on human cells, the αGal epitope is a unique tri-saccharide

Table 2
Differences and similarities between αGal and Neu5Gc related to their immunogenicity in humans

Feature	α-Gal	Neu5Gc
Epitope	Single (Galα1-3Galβ1-4GlcNAc-R)	Multiple
Presence of epitope in human tissues	NO	YES
Biosynthesis in Old World primates	NO	YES
Biosynthesis in humans	NO	NO
Inactive gene in humans	*GGTA1*-KO (α1-3galactosyltransferase)	*CMAH*-KO (CMP-Neu5Ac hydroxylase)
Metabolic incorporation in humans	NO	YES
Humoral response in humans	Anti-Gal IgA, IgG, IgM	Anti-Neu5Gc IgA, IgG, IgM
Level of antibodies in humans	High (in all individuals)	Highly variable (levels and Ig isotypes)
Potential role in diseases related to chronic inflammation	NO*	YES

* αGal may also mediate certain disease condition if it co-exists with the antibodies in the same individual as described in the main text.

Galα1-3Galβ1-4GlcNAc-R that is never presented on human cells. This oligosaccharide can be found at the terminal end of glycoproteins and glycolipids on most mammalian cells except in old world monkeys, apes, and humans.[40] This loss results from a single base deletion leading to frameshift and a premature stop codon in the α1-3GT encoding gene (*GGTA1*) (Table 2).[46,88,92] Therefore αGal is immunogenic in all humans that consequently have circulating anti-Gal IgM, IgA, and IgG with the latter reaching up to 1% of the total serum IgG.[44] Unlike Neu5Gc, αGal cannot be acquired through the diet and it is not expressed on human tissues, yet the high titers of anti-Gal antibodies are due to a continuous stimulation of the gastrointestinal tract by αGal-presenting bacteria such as *Klebsiella pneumonia* and *Escherichia coli*.[45] The co-existence of Neu5Gc/anti-Neu5Gc within humans has clear implications for diseases as described. While αGal/anti-Gal do not normally co-exist in humans, there are certain situations when this does occur and lead to disease conditions. For example, the high anti-Gal titers are an obstacle to xenotransplantation due to expression of αGal in the donor tissues (e.g. pig-to-human)[40] because the anti-Gal antibodies react with the αGal epitope which leads to hyperacute rejection of the graft.[41] This has been described as the anti-Gal/αGal barrier and it prompted the generation of pigs that lack the αGal.[131] Recently, Neu5Gc had also been suggested to negatively affect xenotransplantation.[76,98,126,141] In addition, high titers of anti-Gal antibodies might mediate autoimmune-like responses when an invading pathogen expresses αGal, for example in Chagas disease caused by *Trypanosoma cruzi* that present multiple αGal epitopes on glycoinositolphospholipids and lipophosphoglycans on its cell membrane.[42] On the other hand, the abundant anti-Gal antibodies in humans could be harnessed for therapy that is in cancer therapy or wound healing.[48,161,162] To conclude, αGal and Neu5Gc are two immunogenic carbohydrate moieties in humans that can be used to study potent immune recognition and responses to carbohydrates that are essential for development of novel theranostics.

9. Summary and outlook

This review aimed to provide an overview of immune recognition and response to carbohydrate antigens focusing on the non-human sialic acid Neu5Gc and its unique features compared to the immunogenic αGal. Sialic acids are highly diverse and recent progress in sialoglycans chemoenzymatic synthesis was key for in depth investigation of their various biological roles. This was especially critical to address a century old hypothesis on the role of Neu5Gc in cancer and other diseases in humans. The new glycobiological tools revealed a complicated immune response to diverse Neu5Gc-glycoconjugates that may contribute to chronic inflammation mediated diseases in humans but at the same time holds great promise for designing novel theranostics. Further investigation of the mechanisms allowing immune response to such carbohydrate antigens is critical for rational design of various carbohydrate-based vaccines including cancer vaccines and other novel therapeutic strategies.

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