HUMAN ANTIBODIES AGAINST THE DIETARY NON-HUMAN NEU5GC-CARRYING GLYCANS IN NORMAL AND PATHOLOGIC STATES

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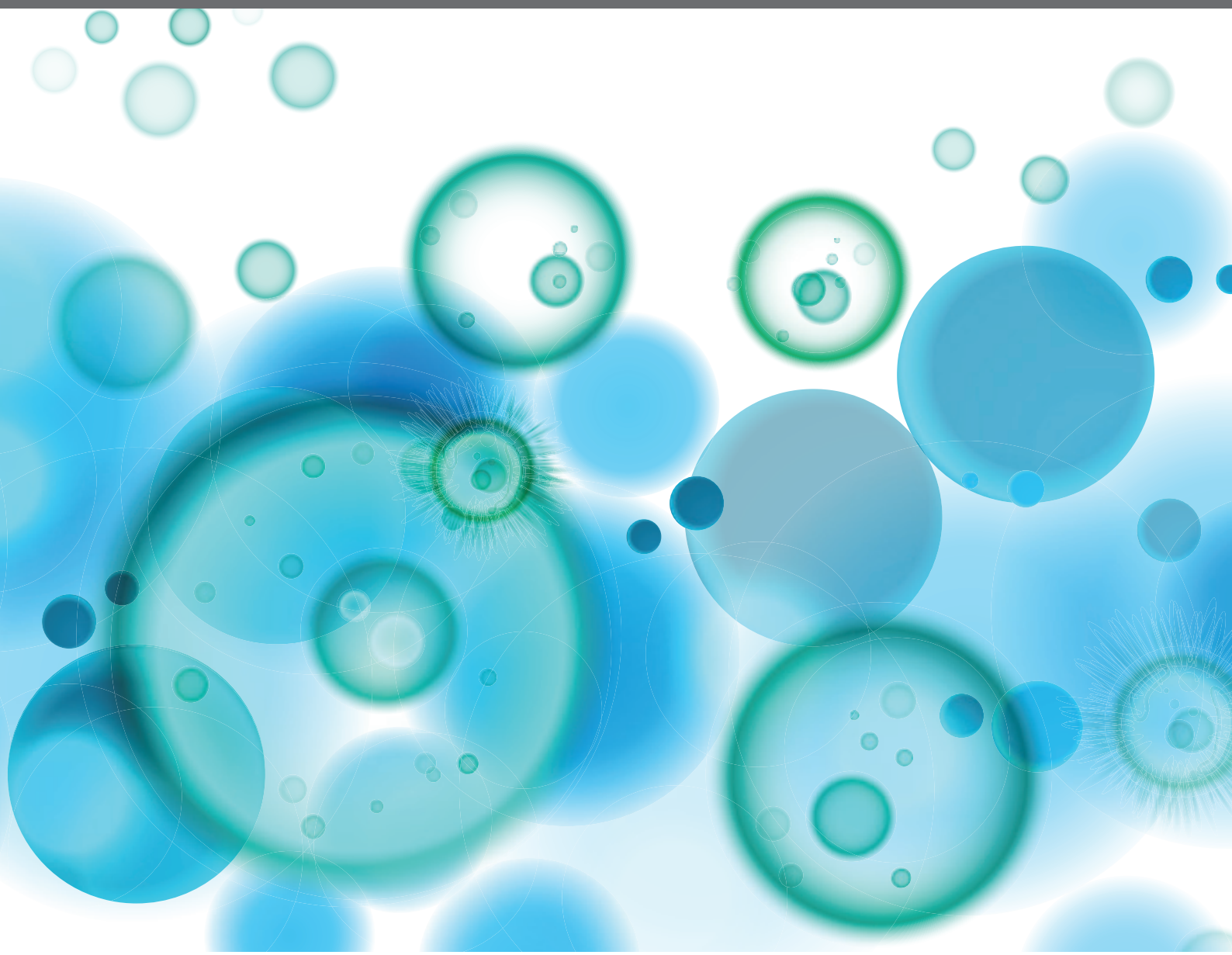

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HUMAN ANTIBODIES AGAINST THE DIETARY NON-HUMAN NEU5GC-CARRYING GLYCANS IN NORMAL AND PATHOLOGIC STATES

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EDITORIAL
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Editorial: Human Antibodies Against the Dietary Non-human Neu5Gc-Carrying Glycans in Normal and Pathologic States

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Editorial on the Research Topic

Human Antibodies Against the Dietary Non-human Neu5Gc-Carrying Glycans in Normal and Pathologic States

While most mammals commonly express the two forms of sialic acids, *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), humans cannot synthesize Neu5Gc due to a loss-of-function mutation in the CMAH gene, which encodes the enzyme responsible for its synthesis. Consequently, Neu5Gc is immunogenic in humans, leading to generation of antibodies against various presentations of Neu5Gc-glycans. These antibodies appear in the first months of life and coincide with dietary intake of Neu5Gc (e.g., red meat and baby formulas containing cow's milk). Co-existence of Neu5Gc and anti-Neu5Gc antibodies in humans may have detrimental consequences, and in recent years, a considerable amount of fundamental information on this subject has been accumulated. Diet-derived Neu5Gc can be absorbed by human cells and can be found at very low levels on the surface of endothelial cells and of oncogenic epithelial cells. This unique situation results in the expression of a non-self carbohydrate in the context of self, coined as a "xeno-autoantigen." Together with circulating anti-Neu5Gc "xeno-autoantibodies," a peculiar "physiological" condition of chronic antibody exposure may lead to *in situ* chronic inflammation, termed xenosialitis, eventually contributing to various human diseases.

This Research Topic embeds a unique collection of papers summarizing current knowledge and remaining open questions regarding the possible consequences of this ongoing immune conflict between anti-Neu5Gc antibodies and Neu5Gc-glycans, during normal and pathological states. The first paper from the Varki group by Dhar et al. provides a historical perspective of this century-old enigma, rooted back in the early days when "serum sickness" was noticed. It also describes possible implications for diseases and therapy (Dhar et al.). Altman and Gagneux provide an evolutionary perspective for the loss of Neu5Gc expression in humans, and Angata puts that into context with host immune recognition of Neu5Gc-neoantigens by the siglecs immune receptors that respond to this evolutionary change. The reader is also referred to the recent historical review by the Center of Molecular Immunology (CIM, Cuba) related to human immunotherapy targeting the GM3(Neu5Gc) ganglioside (1). A major leap forward in research of Neu5Gc/anti-Neu5Gc was achieved owing to the newly developed tools for their investigation. In the paper by Kooner et al. from the Chen group, who pioneered these efforts, describe the chemistry behind the synthesis of designed natural and unnatural glycans with Neu5Gc and their reciprocal human Neu5Ac-containing glycans. Such glycans had been used

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to generate printed glycan microarrays focused on sialic acids that have dramatically advanced the field, as summarized by McQuillan et al. from the Cummings group. Beyond these tools, Breimer and Holgersson provide an overview of the structural complexity, diversity, and distribution of Neu5Gc-glycans in animal tissues, especially in the context of their immunogenicity and implications in clinical xenografting, further extended into the role of anti-Neu5Gc antibodies as an obstacle to xenotransplantation by Tector et al., while Perota and Galli describe the development of Neu5Gc-deficient large mammals (i.e., pigs and bovine) as a possible solution. It is commonly assumed that immune responses against Neu5Gc result from exposure to Neu5Gc-containing food items, however iatrogenic-induction also occur after treatment with Neu5Gc-containing animal-derived biotherpaeutics or bio-devices, yet their possible effects in autoimmune or other diseases remain poorly understood, as described by Yehuda and Padler-Karavani. In addition, Frei et al. discuss how rural lifestyle and increased anti-Neu5Gc antibodies levels could be protective against allergies. Despite this accumulated knowledge thus far, the mechanisms underlying the roles of anti-Neu5Gc antibodies particularly in human pathologies are largely unexplored, and most current investigations focus on several unresolved theoretical and fundamental questions directly related to a possible deleterious role of anti-Neu5Gc antibodies in humans. Thus, Soulillou et al. provide a critical perspective on current literature related to the suggested roles of anti-Neu5Gc antibodies in human pathologies. Altogether, this special issue is a major contribution to increase awareness of this very complex research related to the immunogenic Neu5Gc dietary carbohydrate in humans and its potential involvement with multiple human diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: J-PS is the founder of Xenothera à French start-up dedicated to Neu5Gc knockout pig products, and collaborate with Avantea, a company with which they have produced Neu5Gc knockout cows.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Possible Influences of Endogenous and Exogenous Ligands on the Evolution of Human Siglecs

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Sialic acids, a group of acidic sugars abundantly expressed in the tissues of deuterostome animals but rarely found in microbes, serve as a "signature of self" for these animals. Cognate sensors for sialic acids include Siglecs, a family of transmembrane lectins of vertebrate immune systems that recognize glycans containing sialic acids. A type of sialic acid called *N*-glycolylneuraminic acid (Neu5Gc) is abundant in many mammalian lineages including great apes, the closest extant relatives of modern human, but was lost in the lineage leading to modern human via the pseudogenization of the *CMAH* gene encoding the enzyme that converts *N*-acetylneuraminic acid (Neu5Ac) to Neu5Gc. Loss of Neu5Gc appears to have influenced the evolution of human Siglecs, such as the adjustment of sialic acid binding preferences and the inactivation of at least one Siglec. In addition, various mechanistic studies using model systems and genetic association studies have revealed that some human Siglecs interact with pathogens and influence the outcome of infections, and these pathogens in turn likely influence the evolution of these Siglecs. By understanding the evolutionary forces affecting Siglecs, we shall achieve a better appreciation of Siglec functions, and by understanding Siglec functions, we can obtain deeper insight into the evolutionary processes driving Siglec evolution.

Keywords: Siglec, sialic acid, Neu5Ac, Neu5Gc, immunity, microbes

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INTRODUCTION

The role of immunity is to distinguish self vs. non-self (or what is not dangerous vs. dangerous) and to eliminate or contain the latter. Various biomolecules (nucleotides, peptides, lipids, polysaccharides, and their combinations) can be a signature of non-self (i.e., pathogen-associated molecular patterns; PAMPs), as exemplified by the diversity of ligands for Toll-like receptors, C-type lectin-like receptors, RIG-I-like receptors, and NOD-like receptors, all of which work as "pattern-recognition receptors" (1–4). Meanwhile, the signature of self (i.e., self-associated molecular patterns; SAMPs) is less well-understood, but some glycoconjugates would qualify as such (5, 6). Sialic acids are commonly synthesized by deuterostome animals and displayed on the cell surface in abundance but are rare in microbes (7), making them an ideal SAMP for distinguishing self- vs. non-self (5, 6).

For a chemical entity to be a molecular signature of self or non-self for the immune system, there must be a sensor that recognizes it. For sialic acids, Siglecs appear to be the primary pattern-recognition receptors (8–11). Siglec is a composite word from "sialic acid," "immunoglobulin (Ig) superfamily," and "lectins" (12). The Siglec family appears to be present only in vertebrates (13, 14). Siglecs are type 1 transmembrane proteins, with an extracellular domain consisting of multiple Ig-like domains (of which the N-terminal Ig-like domain is primarily responsible for the recognition of sialoglycans), followed by a single-pass transmembrane domain and cytoplasmic tail (**Figure 1**). Most of the known mammalian Siglecs are expressed on leukocytes and have an intracellular sequence motif called the immunoreceptor tyrosine-based inhibitory motif (ITIM) that recruits tyrosine phosphatase SHP-1 and thus transduces inhibitory signals. Thus, they are considered to function as sensors for sialic acids as a molecular signature of self. [However, there are some examples that imply this generalization may be somewhat too simplistic (17, 18).]. Although rodents are essential model animals for mechanistic studies in immunology, differences in primate and rodent CD33-related Siglecs (15) impose a significant challenge in the extrapolation of findings in rodents to human immunology. This situation parallels that of other immunoglobulin-like receptor families, leukocyte immunoglobulin-like receptors (LILR) and killer cell immunoglobulin-like receptors (KIR), that are encoded in a gene cluster on the same human chromosomal region as CD33-related Siglecs (chromosome 19q13.4) and are involved in self-recognition through interaction with MHC class I (19–21).

"Sialic acids" is a collective term for various naturally occurring acidic sugars with a common nine-carbon backbone (22). *N*-acetylneuraminic acid (Neu5Ac) is the most common type of sialic acid, and its C5-hydroxylated derivative *N*-glycolylneuraminic acid (Neu5Gc), along with the derivatives of Neu5Ac and Neu5Gc (mostly modified at C4 and/or C7-C9 hydroxyl groups), are generally present in mammalian tissues (22). Neu5Gc is abundant in many mammalian species, whereas humans have lost Neu5Gc, owing to the mutation (exon deletion) of the *CMAH* gene encoding CMP-Neu5Ac hydroxylase that is solely responsible for the *de novo* biosynthesis of Neu5Gc from Neu5Ac (23–26). Although some bacteria have developed ways to synthesize Neu5Ac, so far no study has demonstrated the presence of Neu5Gc on microbes (27) [A recent genomic survey (28) reported the presence of *CMAH*-like sequences in several microbial genomes, including those of several *Helicobacter* species that may express sialic acids. However, their enzymatic function has not yet been investigated]. Thus, Neu5Gc appears to be a quintessential signature of self, which is only present on deuterostome cells and missing on microbes. Indeed, some rodent Siglecs show a strong preference toward glycans containing Neu5Gc (29–32), whereas some others show a strong preference toward Neu5Ac (29, 33, 34). This imposes a conundrum: if one loses the best signature of self, the immune system may become more prone to attack its own cells (i.e., autoimmunity). How did the immune system of the human ancestor cope with the consequences of the dramatic change in the sialic acid landscape (i.e., shift of "sialome" from Neu5Gc to Neu5Ac) on the cell surface?

One possible consequence of Neu5Gc loss in human (and a possible response to the consequential autoimmune-prone state, in the evolutionary time scale) was a series of changes involving Neu5Gc-specific Siglecs, such as re-adjustment of binding specificity to Neu5Ac and "forced retirement," as explained in the following section.

POSSIBLE INFLUENCES OF NEU5GC LOSS ON HUMAN SIGLECS: ALTERED BINDING SPECIFICITIES

To understand the consequences of a species-specific event, it is natural to compare the phenotypes between the closest relatives that have undergone the event or have not. For human, the obvious choice is great apes including chimpanzee, which is the closest extant relative of modern human. Several earlier studies have shown that at least some great ape Siglecs preferentially recognize Neu5Gc (35–37). More recent data using the sialoglycan microarray also showed that primate CD33-related Siglecs generally tend to prefer Neu5Gc (38). Reported preferences of human and chimpanzee Siglecs toward Neu5Ac and Neu5Gc are summarized in **Table 1**. Thus, the loss of Neu5Gc likely meant attenuation of the interactions between Siglecs and self-associated ligands in the human ancestor.

One Siglec that may have been substantially affected by the loss of Neu5Gc in the human ancestor is Siglec-12 (36). Chimpanzee Siglec-12 and human Siglec-XII are expressed on macrophages and lumenal epithelia (36, 46). Human Siglec-XII has a universal mutation (R122C) that makes the protein unable to recognize sialic acids (36). [Roman numerals are used for primate Siglecs that have a mutation at the essential arginine residue required for sialic acid recognition and thus cannot recognize sialic acid (15)]. Arginine-restored human Siglec-XII, as well as chimpanzee Siglec-12, strongly prefers Neu5Gc over Neu5Ac (36). In addition, some human *SIGLEC12* alleles have acquired additional mutations (stop codon, rs16982743, and frame-shift, rs66949844) that cause premature termination of Siglec-XII protein synthesis (36, 46). These "null" mutations are common in the modern human populations (global frequency of "null" alleles: 0.19 for rs16982743, 0.59 for rs66949844). These results imply a scenario in which a Siglec that lost an endogenous ligand was forced to "retire" and then is further getting eliminated. Given that the R122C mutation is fixed in modern human populations, it is tempting to speculate that the presence of functional "Neu5Gc-recognizing" Siglec-12 may have caused a disadvantage in ancestral humans. For example, zoonotic infection of some Neu5Gc-coated envelope virus from other mammalian species may represent such selective pressure. A possible scenario for the further elimination of "signal transduction-competent but sialic acid recognition-incompetent" Siglec-XII may be that the recruitment of SHP-2 by Siglec-XII (47) on epithelial cells may assist the transformation of the epithelial cell by an oncogenic driver (e.g., receptor tyrosine kinase or RAS mutation/amplification) through activation of

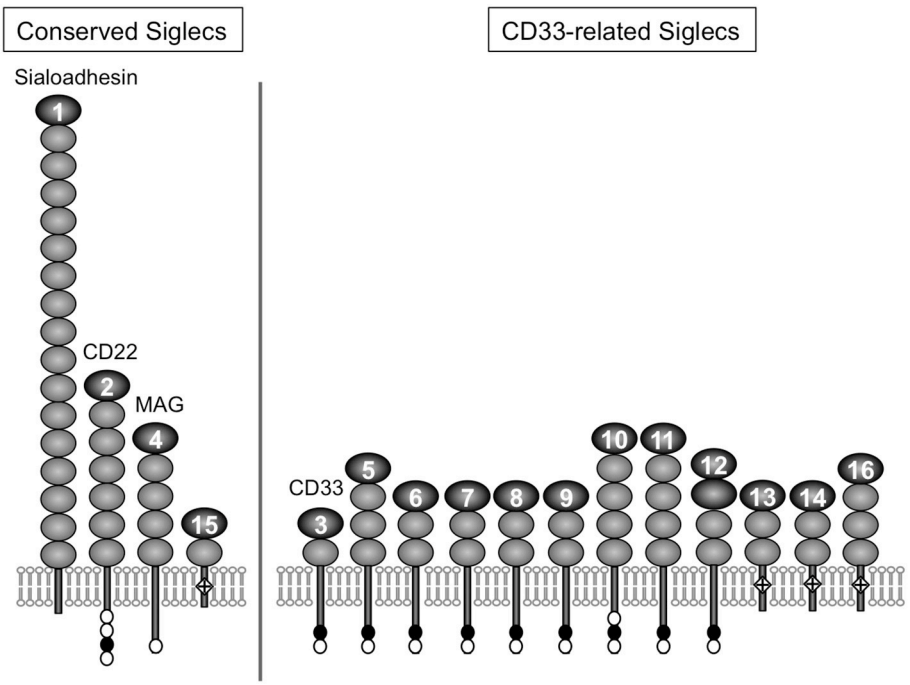

FIGURE 1 | Illustrative representation of ape Siglecs. Mammalian Siglecs can be classified as relatively conserved members (Sialoadhesin/Siglec-1, CD22/Siglec-2, MAG/Siglec-4, and Siglec-15) and less conserved members (CD33-related Siglecs). CD33-related Siglecs are encoded in the gene cluster (15). Siglecs can be also classified based on the partner molecule involved in downstream signal transduction (i.e., those that have ITIM and interact with tyrosine phosphatase SHP-1, and those that have positively charged amino acid residue in the transmembrane domain and interact with adapter molecule DAP12, which has the "immunoreceptor tyrosine-based activating motif" (ITAM) and recruits tyrosine kinase Syk). Siglec-13 is missing in humans but present in apes and old-world monkeys (15). Modified from (16). Closed circle, open circle, and diamond with + mark in the figure represent ITIM, ITIM-like motif, and positively charged amino acid residue in the transmembrane domain (that is required for the interaction with DAP12), respectively.

MAPK pathway (48–52), which may have been disadvantageous for the overall fitness of the carriers of the functional allele. However, at present there is no solid experimental evidence to support these speculations.

Primate Siglec-9 (from chimpanzee, gorilla, and baboon) also prefers Neu5Gc, whereas human Siglec-9 appears to have acquired affinity toward Neu5Ac (37, 38). Human CD33/Siglec-3 and Siglec-5 also show a similar acquired affinity to Neu5Ac compared with their counterparts in baboon, which show a strong preference for Neu5Gc (38). Given that Siglec-9 has an ortholog in rodents (Siglec-E), it may play an important role in regulating innate immunity and be indispensable (although expression patterns and functions of primate Siglec-9 and rodent Siglec-E may not completely overlap (53–55)). Human Siglec-9 may have had to undergo rapid evolution to catch up with the change in the human sialome, to resume its original functionality. It is of note that the N-terminal Ig-like domain (Ig1) of great ape Siglec-9 shows much greater inter-species sequence differences than does the adjacent C2-set Ig-like domain (Ig2) (37), which is consistent with the idea that human Siglec-9 had to evolve rapidly to respond to the loss of Neu5Gc.

In fact, the CD33-related Siglec gene cluster is among the most rapidly diversifying gene families between human and chimpanzee (56), and the N-terminal Ig-like domain of CD33-related ape Siglecs is evolving faster than the other parts of the molecule (15, 37, 57). It is of interest whether the loss of Neu5Gc contributed to the accelerated evolution of human Siglecs. Assuming this is the case, we would expect that more amino acid changes have accumulated in the first Ig-like domain (Ig1) of human Siglecs than in Ig1 of chimpanzee Siglecs. In reality, the data (**Table 1**) do not appear to support this prediction. Although it is true that Ig1 is undergoing faster evolution than Ig2 (total human-specific changes in Ig1 and Ig2: 33.5 and 15, respectively; total chimpanzee-specific changes in Ig1 and Ig2: 40.5 and 14, respectively; average amino acid length of Ig1 and Ig2: 126 and 96, respectively), the Ig1 of Siglecs in the lineage leading to human has accumulated less sequence changes than that leading to chimpanzee. Some of the sequence changes in human Siglecs probably represent a genuine sign of selection due to the loss of Neu5Gc, whereas the majority of them may not be. Ig1 of primate Siglecs (and likely those in other species) is evolving rapidly under selective pressure that may include, but not be limited to, the changes in the landscape of endogenous sialoglycans (38).

It is of note that, in contrast to the adaptation of some human Siglecs to Neu5Ac-dominant sialome, many Siglecs still appear to prefer Neu5Gc (**Table 1**). Possible explanations for this fact may include: (1) the adaptation of human Siglecs

TABLE 1 | Binding preferences (Neu5Ac vs. Neu5Gc) and lineage-specific mutations in human and chimpanzee Siglecs.

Siglec	Human preference	Chimpanzee preference	Human-specific changes in Ig1 and Ig2*	Chimpanzee-specific changes in Ig1 and Ig2*	References**
Sialoadhesin/Siglec-1	**Ac** >> Gc	ND	1, 1	1, 0	(39)
CD22/Siglec-2	Ac ≈ Gc	Ac ≈ Gc	2, 0	2, 0	(35, 39)
CD33/Siglec-3	Ac < **Gc** (weak preference)	Ac < **Gc** (weak preference)	2.5, 2	5.5, 0	(35, 38, 39)
MAG/Siglec-4	ND	ND	0, 1	0, 0	[for the glycan preference of rodent MAG, see (33, 34, 40, 41)]
Siglec-5	Ac < **Gc** (weak preference)	X (Arg mut)	7.5, 2	5.5, 1	(35, 38)
Siglec-6	Ac < **Gc**	ND	0, 1	3, 0	(35)
Siglec-7	Ac ≈ Gc	Ac < **Gc**	1.5, 0	3.5, 0	(37)
Siglec-8	ND	ND	0.5, 2	1.5, 3	[for the glycan preference of human Siglec-8, see (42)]
Siglec-9	**Ac** > Gc (weak preference)	Ac < **Gc** (weak preference)	4, 0	3, 1	(37, 38)
Siglec-10	Ac < **Gc**	ND	0, 1	2, 1	(Consortium for Functional Glycomics data)
Siglec-11	Ac < **Gc**	Ac < **Gc**	2.5, 1	2.5, 0	(43, 44)
Siglec-12	X (Arg mut)	Ac << **Gc**	2, 2	1, 3	(36)
Siglec-13	X (absent)	Ac ≈ Gc	(Cannot be determined)	(Cannot be determined)	(45)
Siglec-14	ND (likely Ac < **Gc**, from Siglec-5 data)	X (Arg mut)	6.5, 1	4.5, 1	(38)
Siglec-15	**Ac** > Gc	ND (**Ac** > Gc)	2, 1	0, 1	Unpublished
Siglec-16	**Ac** > Gc	Ac < **Gc**	1.5, 0	5.5, 3	(43, 44)

ND, not determined; X, cannot be determined (either the protein is absent in the species or is present but does not recognize sialic acid owing to the mutation of essential Arg residue); >> or <<, strong preference; > or <, preference; ≈, no preference; Arg mut: mutation of arginine residue that is essential for sialic acid recognition. Sialic acid (Ac, Neu5Ac; Gc, Neu5Gc) preferentially recognized by each Siglec is highlighted with underline and bold typeface.

**The numbers of human- and chimpanzee-specific amino acid changes were deduced by aligning the amino acid sequences of Siglec orthologs from human, chimpanzee, and orangutan. In case the lineage specificity of the amino acid change cannot be unambiguously determined (i.e., when the amino acid at one position was different in all three species), "0.5 difference" was assigned to both human and chimpanzee. For Siglec-12 with two V-set domains, amino acid changes in the N-terminal V-set domain (Ig1) were counted as those in "Ig1," and those in the first C2-set domain (Ig3) were counted as those in "Ig2." Note that the "species-specific changes" were counted based on a reference sequence of human Siglecs and "best hit" putative protein sequences in chimpanzee and orangutan by BLASTP search, without considering the polymorphisms in each species.*

***The majority of the references in this table are reports that directly compare human and chimpanzee Siglec binding preferences. Note that different methods for analyzing Siglec–glycan interactions, such as glycan microarray vs. polymer-based probe binding, or even between different formats of glycan microarrays, may yield results that are not fully consistent in some cases.*

to Neu5Ac-dominant sialome is still incomplete, and over the time (in the scale of millions of years) most human Siglecs will eventually acquire Neu5Ac preference; (2) some Siglecs did not have strong preference toward Neu5Gc over Neu5Ac prior to the loss of Neu5Gc in human ancestor, or have already accumulated mutations to make them sufficiently suitable for Neu5Ac recognition, thus it is not necessary for them to adapt further to Neu5Ac-dominant sialome; (3) the interaction of Siglecs with exogenous ligands (e.g., bacterial nonulosonic acids) prevent complete switch from Neu5Gc to Neu5Ac preference. Although these explanations are purely speculative, some of these scenarios may be tested experimentally. For example, an independent event has eliminated Neu5Gc in the lineage leading to New World monkeys approximately 30 million years ago (58). In contrast, the timing of Neu5Gc loss in human is far more recent, which is estimated to be 3 million years ago (26). It would be interesting to see whether the Siglecs in New World monkeys prefer Neu5Ac, or some of them still prefer Neu5Gc, to test the validity of the explanation (1) above.

POSSIBLE INFLUENCES OF NEU5GC LOSS ON HUMAN SIGLECS: ALTERED EXPRESSION PATTERNS

It is of interest to know whether there is any change in the expression patterns of Siglecs between human and chimpanzee, which might also represent a consequence of Neu5Gc loss in human. Antibody-based comparative analyses of Siglec expression patterns in human and chimpanzee (and gorilla) have revealed several examples of altered expression of Siglecs in human, as summarized in **Table 2**. Naturally, it is more difficult to establish the influence of the loss of Neu5Gc on the expression patterns of Siglecs than its effect on the binding preferences of Siglecs, as it is indirect. Nevertheless, it appears to be implied in some cases.

The first reported example of altered expression of Siglec in human compared with chimpanzee was the wider distribution of Sialoadhesin/Siglec-1^{+} macrophages in chimpanzee spleen as compared with those in human spleen (39). Although

TABLE 2 | Expression patterns of human and chimpanzee Siglecs.

Siglec	Human	Chimpanzee	References
Sialoadhesin/Siglec-1	Mac	Mac (broader)	(39)
CD22/Siglec-2	B	B (mRNA)	(39)
CD33/Siglec-3	Mono, Mac (broader), Microglia	Mono, Mac, Microglia	(38)
MAG/Siglec-4	Schwann cells, Oligodendroglia	(Myelin)	(59, 60)
Siglec-5	Neutro, Mac (broader), B (low), **amniotic epithelium**	Neutro, Mac, **T**, B	(38, 61–63)
Siglec-6	B, DC subset, **placenta**	B	(64)
Siglec-7	NK, Mono, Mast, Neutro, Baso, Platelets, T (subset)	ND	(65–70)
Siglec-8	Eosino, Baso, Mast	ND	(71, 72)
Siglec-9	Neutro, Mono, Mac (broader)	Neutro, Mono, Mac	(38)
Siglec-10	B, Mono, DC	ND	(73, 74)
Siglec-11	Mac, **Microglia**, ovarian fibroblasts	Mac, ovarian fibroblasts	(43, 75–77)
Siglec-12	Mac, lumenal epithelia	Mac, lumenal epithelia	(36, 46)
Siglec-13	X (absent)	Mono	(45)
Siglec-14	Neutro, Mono, **amniotic epithelium**	Neutro (& Mono?)	(62, 63)
Siglec-15	OC, Mac subset	ND	(78–81)
Siglec-16	Mac, **Microglia**	Mac	(43, 75, 82)

Mac, macrophage; Mono, monocytes; B, B cells; T, T cells; NK, natural killer cells; Mast, mast cells; Neutro, neutrophils; Eosino, eosinophils; Baso, basophils; DC, dendritic cells; OC, osteoclasts; ND, not determined.

**Tissue/cell type that showed clear difference in Siglec expression between human and chimpanzee are highlighted with underline and bold typeface. Reports that directly compared human and chimpanzee Siglec expression patterns are primarily cited in this table. For human Siglecs, expression in the cell types not listed in the table are also reported, such as: CD22/Siglec-2 on basophils (83); CD33/Siglec-3 on mast cells (84), basophils and neutrophils (low) (85); Siglec-6 on mast cells (86); Siglec-9 on T cell subset (68) and NK cell subset (55). Note that the expression of Siglec-6 on human B cells is restricted to CD27$^+$ memory B cells (87). Tumor-infiltrating T cells express several human Siglecs, including CD33/Siglec-3, Sigec-5, Siglec-7, Siglec-9, and Siglec-10 (88).*

the binding specificity of chimpanzee Sialoadhesin/Siglec-1 has not been analyzed, given that both human and mouse Sialoadhesin/Siglec-1 preferentially recognize Neu5Ac (39) and the sequence differences between human and chimpanzee Sialoadhesin/Siglec-1 are small (**Table 1**), it is likely that chimpanzee Sialoadhesin/Siglec-1 prefers Neu5Ac. Thus, the altered distribution of human Sialoadhesin/Siglec-1$^+$ macrophages may be a consequence of the loss of Neu5Gc in humans (39). It is possible that the altered distribution of Sialoadhesin/Siglec-1$^+$ macrophages may be more relevant to the increased density of Neu5Ac in human tissues that may influence the migration of macrophages, rather than a change in cell types that express Sialoadhesin/Siglec-1. In this regard, it would be interesting to know whether the distribution of Sialoadhesin/Siglec-1$^+$ macrophages in *Cmah* knockout mice is different from that in wild-type mice.

One of the most striking changes in Siglec expression patterns in the human immune system is the near-complete absence of Siglec-5 on human T cells, in contrast to its prominent expression on chimpanzee and gorilla T cells (61, 62). The loss of Siglec-5 from human T cells appears to be correlated with the relative hyper-activation of human T cells in response to various stimuli compared with those from other great apes. [Although Siglec-5 and Siglec-14 show extremely high sequence similarity at the extracellular domain, one study (62) used a combination of antibodies that distinguish Siglec-5 and Siglec-14 to demonstrate that Siglec-5 is expressed on chimpanzee T cells]. However, it is not clear whether the loss of Siglec-5 on human T cells has a causative relationship with the loss of Neu5Gc, as human Siglec-5 does not show strong preference for either Neu5Ac or Neu5Gc (38), and its great ape counterparts have a mutation at the essential arginine residue and lack the ability to recognize sialic acids (15, 89). It is also worth mentioning that a recent work demonstrated that Siglec-5 is inducibly expressed by the activation of human T cells (88).

Siglec-11 and Siglec-16 also have undergone unique changes in their expression patterns in humans. Whereas, human Siglec-11 and Siglec-16 are expressed on brain microglia and tissue macrophages, chimpanzee Siglec-11 and Siglec-16 appear to be absent on microglia (but present on tissue macrophages) (43, 75). The change in expression patterns appears to be a consequence of a partial gene conversion of *SIGLEC11* by *SIGLEC16*. Of note, *SIGLEC16* in humans has functional and non-functional alleles (82), and the non-functional allele appears to be the one that converted *SIGLEC11* (90). *SIGLEC11* and *SIGLEC16* have undergone a complex series of concerted evolution through gene conversions in human lineage (90) and also in other lineages of apes (44). Both human and chimpanzee Siglec-11 and Siglec-16 appear to prefer Neu5Gc over Neu5Ac (43, 44), and thus it is tempting to speculate that the loss of Neu5Gc may have had some influence on the altered expression patterns of these Siglecs. Although it is known that the Neu5Gc level is extremely low in mammalian brains (91), Siglec-11 and Siglec-16 also preferentially recognize α2-8–linked Neu5Ac dimers, which are abundant in the brain and serve as ligands for these Siglecs on human microglia.

Siglec-6 was also reported to show different expression patterns between human and chimpanzee. Both human and

chimpanzee Siglec-6 are expressed on B cells, whereas its expression on placental trophoblasts is observed only in humans (64). This altered expression is thought to be associated with the sequence change in the promoter region and transcription factor binding (64).

There are some reports of the presence of Siglec ligands in human tissues that are absent in chimpanzee tissues (64, 76). Although the exact nature of these ligands has not been identified, these findings imply that the difference in Siglec ligand expression patterns beyond the absence/presence of Neu5Gc may exist between human and chimpanzee and may also contribute to the rapid evolution of the Siglec family (particularly at Ig1) and/or their altered expression patterns.

AN ALTERNATIVE DRIVING FORCE BEHIND SIGLEC EVOLUTION: INTERACTION WITH MICROBES

Given that Ig1 of Siglecs (particularly that of CD33-related Siglecs) is undergoing rapid evolution (57), and not all of this may be attributed to the changing endogenous ligand landscape, there is likely an alternative driving force behind their rapid evolution. Obviously, one such force could be microbial pathogens that engage Siglecs. Indeed, recent studies have provided evidence that many Siglecs are involved in the interaction with various pathogenic microbes [for recent reviews, see (92, 93)]. These microbes include viruses, bacteria, and eukaryotic pathogens (**Table 3**). Many of them cover themselves with sialic acids (either by *de novo* biosynthesis or by "salvage" from the human body by various mechanisms), which may be considered examples of "molecular mimicry" by microbes.

The majority of the microbes reported to interact with Siglecs so far are bacteria (**Table 3**). This makes sense, as sialic acids (and sialic acid-like nonulosonic acids) are occasionally found in bacterial extracellular components, such as lipopolysaccharides/lipooligosaccharides (LPS/LOS), capsular polysaccharides (CPS), and flagella. For example, group B streptococcus (GBS) type III interacts with Siglec-9 through sialylated CPS and dampens inflammatory responses by neutrophils (98), whereas GBS type Ia engages Siglec-5 by β-protein and also suppresses inflammatory responses of myeloid cells (100). It should be noted that the latter case does not involve sialic acids. Similarly, non-typeable *Haemophilus influenzae*, an opportunistic airway pathogen, engages Siglec-5 and attenuates pro-inflammatory cytokine production by myeloid cells (102), and *Escherichia coli* K1 strain, a neurotropic pathogen, engages Siglec-11 and escape killing (75). Siglecs are likely under pressure to escape the exploitation by these pathogens, which may partially explain the driving force behind their rapid evolution.

It appears that Siglecs were not just escaping from these pathogens; they appear to have developed "counter-traps" against these pathogens. Some Siglecs (i.e., Siglec-5 and Siglec-14; Siglec-11 and Siglec-16) are found to be "paired receptors," which are two Siglecs with highly homologous extracellular domains recognizing similar ligands, combined with intracellular signaling modules transducing opposing signals (i.e., one of the pair interacts with SHP-1 and transduces the inhibitory signal, whereas the other interacts with adapter protein DAP12 and tyrosine kinase Syk and transduces the activating signal). In fact, whereas the engagement of inhibitory Siglec by pathogenic bacteria suppresses anti-bacterial responses, the engagement of activating Siglec counteracts this effect (63, 75, 102). It is of note that these "paired" Siglecs appear to show more sequence differences between human and chimpanzee than other "stand-alone" Siglecs (**Table 1**), possibly implying that these Siglecs are under higher selective pressure to diversify than are other Siglecs. These paired Siglecs are undergoing concerted evolution through repeated gene conversions (43, 44, 89, 90), which is likely necessary to maintain the effectiveness of activating-type Siglec as "counter-traps." It is also intriguing that, in humans, null alleles for these activating-type Siglecs (Siglec-14 and Siglec-16) are found at very high frequencies (82, 113).

Evidence supporting the relevance of these interactions between Siglecs and bacterial pathogens in infectious diseases is emerging from genetic association studies (**Table 4**). Small-scale case–control studies investigating the possible correlations between the polymorphisms of *SIGLEC* genes and infectious disease susceptibility have revealed some correlations, such as *SIGLEC14* null polymorphism and COPD exacerbation (102), pre-term delivery in the presence of GBS (63), *Mycobacterium tuberculosis* meningitis (138), and *SIGLEC9* polymorphism and COPD exacerbation (133). In addition, large-scale genome-wide association studies (GWAS) have also revealed possible associations between *SIGLEC* polymorphisms and infectious diseases, such as *SIGLEC5* polymorphism and leprosy (129) and severe periodontitis (128), although these GWAS did not demonstrate a direct interaction between the etiological agents and Siglec protein. Some *SIGLEC* genetic polymorphisms appear to influence the leukocyte counts (142); thus it is possible that the influence of *SIGLEC* genetic polymorphisms on antibacterial defense may be indirect. Regardless, the application of GWAS to infectious diseases may further reveal the relevance of Siglecs for immunological defense against bacterial pathogens.

With regard to viral pathogens, recent studies have revealed that Sialoadhesin/Siglec-1 (also known as CD169) may play a major role in retrovirus infection (103). For example, several groups have reported that human immunodeficiency virus (HIV) exploits Sialoadhesin/Siglec-1 to enhance infection of $CD4^+$ T cells (the primary target cells) by trans-infection (i.e., the virus particle is captured by macrophages with Sialoadhesin/Siglec-1, which transfers the virus to $CD4^+$ T cells and facilitates the infection) (104–107). Although a rare "null" mutation in the *SIGLEC1* gene was found not to protect carriers from HIV infection (144), the low frequency of this mutation (allele frequency: ∼1.3% in Europeans) may preclude us from making a definitive conclusion (145). Given that Sialoadhesin/Siglec-1 appears to be involved in retroviral infection in both mouse and human (103), one may expect that it should evolve rapidly to avoid viral infections; however, Sialoadhesin/Siglec-1 does not appear to be evolving rapidly (**Table 1**). This may be because enveloped viruses are coated with a host-derived membrane (a

TABLE 3 | Direct interaction of human Siglecs and microbes.

Microbe	Microbial molecule involved	Human siglec involved	Outcome	References
BACTERIA				
Neisseria meningitidis	Sialic acids on LPS	Sialoadhesin/Siglec-1 Siglec-5	Enhanced binding and phagocytosis	(94)
Campylobacter jejuni	Sialic acids on LPS	Sialoadhesin/Siglec-1 Siglec-7	Modulation of factors affecting helper T-cell differentiation	(95–97)
	Pseudaminic acid on flagellin	Siglec-10	Promote anti-inflammatory response	(74)
Group B Streptococcus type III	Sialic acids on CPS	Siglec-9	Attenuated immune responses	(98, 99)
Group B Streptococcus type Ia	β protein (Sia-independent)	Siglec-5 Siglec-14	Siglec-5: Attenuated responses Siglec-14: Enhanced responses	(63, 100)
		Siglec-13 (chimpanzee)	Attenuated response	(45)
Pseudomonas aeruginosa	Sialic acids on glycoproteins, adsorbed from human body fluid	Siglec-9	Attenuated immune responses	(101)
Non-typeable *Haemophilus influenzae*	Sialic acids on LOS + Sia-independent interaction	Siglec-5 Siglec-14	Siglec-5: Attenuated responses Siglec-14: Enhanced responses	(102)
Escherichia coli K1 strain	CPS (polysialic acids)	Siglec-11 Siglec-16	Siglec-11: Attenuated responses Siglec-16: Enhanced responses	(75)
VIRUSES				
Human immunodeficiency virus (HIV)	Sialic acids on gp120 envelope glycoprotein; host-derived gangliosides on envelope	Sialoadhesin/Siglec-1 Siglec-7	Enhanced infection	(103–108)
Varicella zoster virus (VZV), herpes simplex virus (HSV)	Glycoprotein B (sialic acids required)	MAG/Siglec-4	Enhanced infection	(109, 110)
EUKARYOTES				
Candida albicans	zymosan (?)	Siglec-7	Enhanced immune responses	(111)
Leishmania donovani	Surface sialic acids	Sialoadhesin/Siglec-1 Siglec-5	Enhanced infection	(112)

Updated from Angata and Varki (93).

part of "self"), and thus there is no way Sialoadhesin/Siglec-1 can evolve to completely evade such an interaction (unless the virus develops a protein that binds Sialoadhesin/Siglec-1 in sialic acid-independent manner). It is worth noting that myelin-associated glycoprotein (MAG)/Siglec-4, the other Siglec known to interact with another enveloped virus (109, 110), is also highly conserved among mammals, and in both cases sialic acids are required for the interaction between the virus and Siglecs (**Table 3**).

CONCLUSION AND PERSPECTIVES

Cmah null mouse is a valuable tool for the investigation of the physiological roles of Neu5Gc and the short-term consequences of its loss, although it may not be a perfect model of modern human. Using this mouse model, it was shown that the expression of Neu5Gc itself makes T cells less responsive to stimulus, without any change in Siglec expression (146). Likewise, Neu5Gc appears to have a general suppressive effect on mouse monocyte/macrophage activities, without the apparent involvement of Siglecs (147). In line with these findings, the loss of Neu5Gc has had major influences on human biology that reach far beyond Siglecs (148), explaining some of the differences between human and our close relatives (e.g., chimpanzee) in pathophysiological phenotypes (149). Although Neu5Gc from dietary sources (in the form of meat or milk from the animals that express Neu5Gc) can be incorporated into human tissue glycoproteins and glycolipids (150), the level of Neu5Gc in human tissues tends to be low, accounting for <1% of total sialic acids (151). Given that the current set of human Siglecs lack a strong preference toward Neu5Gc, and the affinity between Siglecs and sialic acids tends to be low (K_d in ~mM range), this level of Neu5Gc in human tissues may not influence human physiology by way of Siglecs. The trace amount of Neu5Gc

TABLE 4 | Polymorphisms in human *SIGLEC* genes and association with disease/phenotype.

Gene	Polymorphism	Associated phenotype	References
SIGLEC1	rs656635, rs609203, rs3859664, rs4813636 (SNPs in intron or 3'UTR)	Lung function	(114)
SIGLEC1	rs6037651 (nonsynonymous SNP)	Serum IgM level	(115)
CD22	rs34826052 (synonymous SNP)	Limited cutaneous systemic sclerosis	(116)
CD22	rs4805119 etc. (intronic SNP)	B-precursor leukemia	(117, 118)
CD33	rs3865444 (promoter SNP) rs12459419 (nonsynonymous SNP, influencing splicing)	Late-onset Alzheimer's disease	(119–122)
CD33	rs35112940, rs12459419 (nonsynonymous SNPs)	Efficiency of antibody therapy in pediatric acute myeloid leukemia	(123, 124)
MAG	rs720309 (intronic SNP) rs7249617 (intronic SNP)	Schizophrenia	(125, 126) (127)
SIGLEC5	rs4284742 (intronic SNP)	Periodontitis	(128)
SIGLEC5	rs10414149 (intronic SNP)	Leprosy	(129)
SIGLEC6	rs2305772 (non-synonymous SNP, influencing splicing)	Systemic lupus erythematosus	(130)
SIGLEC8	rs36498 (promoter SNP) rs10409962 (nonsynonymous SNP)	Allergic asthma	(131)
SIGLEC9	rs16988910 (nonsynonymous SNP)	Short-term survival of lung cancer patients; Emphysema	(132)
SIGLEC9	rs2075803, rs2258983 (nonsynonymous SNP)	COPD exacerbation	(133)
SIGLEC11	rs12165127 (intronic SNP)	Lung cancer in never-smokers	(134)
SIGLEC12	rs16982743 (stop codon generated)	Cardiovascular outcomes in patients with hypertension on antihypertensive therapy	(135)
SIGLEC12	rs3752135 (nonsynonymous SNP)	Stress fracture	(136)
SIGLEC14	rs10412972, rs11084102 (upstream SNPs)	Plasma plasminogen level	(137)
SIGLEC14	*SIGLEC14-SIGLEC5* fusion (*SIGLEC14* deletion)	COPD exacerbation	(102)
SIGLEC14	*SIGLEC14-SIGLEC5* fusion (*SIGLEC14* deletion)	Pre-term delivery in the presence of GBS infection	(63)
SIGLEC14	*SIGLEC14-SIGLEC5* fusion (*SIGLEC14* deletion)	*Mycobacterium tuberculosis* meningitis	(138)
Various	Various	Plasma protein levels	(139, 140)
Various	Various	Cerebrospinal fluid protein levels	(141)
Various	Various	Blood cell counts	(142)

Updated from Angata (16) and Angata (143).
Some of the studies listed above are small-scale case–control studies, whereas some others are large-scale genome-wide association studies (GWAS). Some of the associations listed are not prominently featured in the references cited but found in the GWAS catalog (https://www.ebi.ac.uk/gwas/).

incorporated into human tissues may be more relevant to the bacterial toxins that specifically recognize Neu5Gc (152) and xeno-autoantibodies that recognize Neu5Gc (as discussed in other articles of this series). Regardless, the loss of Neu5Gc appears to have left some footprint on the evolution of human Siglecs, as discussed above.

The evolution of human Siglecs was also likely influenced by the interaction with microbes. A recent population genetics-based study implied that some Siglecs may have been subjected to population-specific hard selective sweeps, as judged by the presence of long-range linkage disequilibrium (153). These *SIGLEC* genes include *SIGLEC8* and *SIGLEC10* among Africans, *SIGLEC5*, *SIGLEC6*, *SIGLEC12*, and *SIGLEC14* among Europeans, and *CD22* and *MAG* among Asians. Although it remains speculative, the population-specific difference in the signatures of selection imply that the evolution of the Siglec family in the human population is an ongoing process, and different pathogen pressures are present in different geographical locations (or through different agricultural constraints, e.g., use of different domestic animals, which may carry different kinds of bacteria/viruses).

Many questions remain with regard to the function and evolution of Siglecs. For example, do viruses really target only conserved Siglecs and are they not relevant to the rapid evolution of Siglecs? What was or is the selective force behind the spread of "null" alleles of *SIGLEC14* and *SIGLEC16* (and perhaps others, such as *SIGLEC1*) in modern human populations? Do the bacteria that express sialic acid-like nonulosonic acids (154) generally engage Siglecs to modulate immune responses and thus play a role in the evolution of Siglecs? Does the interaction between Siglecs and commensal bacteria (e.g., normal gut microbiota) play any role in the modulation of immunity and the evolution of Siglecs? Some of these questions can be addressed experimentally and will deepen our understanding of the biology of Siglecs and sialic acids.

AUTHOR CONTRIBUTIONS

TA analyzed the literature and wrote the manuscript.

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REVIEW
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From "Serum Sickness" to "Xenosialitis": Past, Present, and Future Significance of the Non-human Sialic Acid Neu5Gc

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The description of "serum sickness" more than a century ago in humans transfused with animal sera eventually led to identification of a class of human antibodies directed against glycans terminating in the common mammalian sialic acid *N*-Glycolylneuraminic acid (Neu5Gc), hereafter called "Neu5Gc-glycans." The detection of such glycans in malignant and fetal human tissues initially raised the possibility that it was an oncofetal antigen. However, "serum sickness" antibodies were also noted in various human disease states. These findings spurred further research on Neu5Gc, and the discovery that it is not synthesized in the human body due to a human-lineage specific genetic mutation in the enzyme *CMAH*. However, with more sensitive techniques Neu5Gc-glycans were detected in smaller quantities on certain human cell types, particularly epithelia and endothelia. The likely explanation is metabolic incorporation of Neu5Gc from dietary sources, especially red meat of mammalian origin. This incorporated Neu5Gc on glycans appears to be the first example of a "xeno-autoantigen," against which varying levels of "xeno-autoantibodies" are present in all humans. The resulting chronic inflammation or "xenosialitis" may have important implications in human health and disease, especially in conditions known to be aggravated by consumption of red meat. In this review, we will cover the early history of the discovery of "serum sickness" antibodies, the subsequent recognition that they were partly directed against Neu5Gc-glycans, the discovery of the genetic defect eliminating Neu5Gc production in humans, and the later recognition that this was not an oncofetal antigen but the first example of a "xeno-autoantigen." Further, we will present comments about implications for disease risks associated with red meat consumption such as cancer and atherosclerosis. We will also mention the potential utility of these anti-Neu5Gc-glycan antibodies in cancer immunotherapy and provide some suggestions and perspectives for the future. Other reviews in this special issue cover many other aspects of this unusual pathological process, for which there appears to be no other described precedent.

Keywords: Neu5Gc, anti-Neu5Gc, serum sickness, xenosialitis, red meat, sialic acid, inflammation, antibodies

FIRST REPORTS OF "SERUM SICKNESS" IN HUMANS INFUSED WITH ANIMAL SERUM

Following the discovery of the effectiveness of tetanus and diphtheria antitoxins by Emil von Behring and Shibasaburo Kitasato, the popularity of serotherapy soared in the 1880s and 1890s (1). However, reports of reactions to the diphtheria antitoxin also started to appear. In 1899, Bolton reported 100 cases of reactions to the diphtheria antitoxin (2). Pirquet and Schick suggested the use of the phrase "serum sickness" in their book *Die Serumkrankheit* (3) recognizing that the reactions were against animal serum components present in the antitoxin preparations.

"SERUM SICKNESS" PATIENTS HAVE "H-D" ANTIBODIES, SOME OF WHICH RECOGNIZE NEU5GC-CONTAINING GLYCANS FOUND IN HUMAN CANCERS

The Initial Definition of "H-D" Antibodies

Two decades later, Hanganutziu and Deicher independently described human antibodies that agglutinated animal erythrocytes (4, 5). These Hanganutziu-Deicher antibodies (H-D antibodies) were prominent in subjects with serum sickness who had received therapeutic animal antisera. Subsequently, similar antibodies were reported in patients with no prior exposure to animal sera but instead suffering from other diseases (6).

A Portion of H-D Antibodies Are Directed Against Neu5Gc-Containing Glycans, but HD Antigens Can Also Be Present in Diseased Human Tissues

About 50 years later, two groups independently showed that a portion of these heterophile H-D antibodies recognized gangliosides containing the sialic acid *N*-Glycolylneuraminic acid (Neu5Gc) (7, 8). This sialic acid was later shown to be derived from the common mammalian sialic acid *N*-Acetylneuraminic acid (Neu5Ac) by the addition of a single oxygen atom that is added to CMP-Neu5Ac in a complex cytosolic reaction catalyzed by the enzyme cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (*Cmah*) (9–13). The definition of H-D antibodies sparked further research and these were then detected in the sera of patients with multiple pathological conditions, including rheumatoid arthritis, infectious mononucleosis, leprosy, syphilis, leukemia, Kawasaki disease (a disease that causes inflamed blood vessels), and various cancers (14–24).

Generation of H-D Antibodies in Chickens, Confirming H-D Antigens in Human Cancers

Early on, it was also noted that anti-H-D serum of high titer could be generated in chickens immunized with H-D antigen-active glycosphingolipid, *N*-Glycolylneuraminyl-lactosylceramide (purified from equine erythrocytes) (18, 25). Immunohistochemistry or thin-layer chromatography using these polyclonal antibodies as well as indirect methods such as inhibition of bovine erythrocyte agglutination by human H-D antiserum were then used to confirm the presence of Neu5Gc-glycans in meconium and multiple human tumors (14–24). Paradoxically, the H-D antigens or Neu5Gc-glycans were also found on human tissue gangliosides and glycoproteins (18, 25–35). Much later, work from our group resulted in further affinity purification of such chicken polyclonal antibodies (36) (during the process we have noted that the bovine serum albumin preparation originally used as a "carrier" for the immunogen is contaminated with bovine serum glycoproteins bearing Neu5Gc-glycans, which also contribute importantly to the immune response in chickens). These preparations were used as a valuable tool for the detection of smaller amounts of Neu5Gc-glycans present even in normal human tissues (36, 37), particularly on epithelia lining hollow organs (the origin of carcinomas), and on endothelia (where atherosclerotic cardiovascular disease occurs).

HUMANS CANNOT SYNTHESIZE Neu5Gc

Humans Are Genetically Deficient in *CMAH*, the Primary Enzyme That Generates Neu5Gc

These findings inspired further work on *CMAH*, and the discovery of an inactivating mutation that likely got fixed in the human lineage >2 million years ago. All humans were found to be homozygous for a deletion of exon 6 in the *CMAH* gene (38, 39) and this deletion was later shown to have been mediated by a single Alu-Alu fusion event (40). While the first published report incorrectly claimed that the mutation resulted in an altered reading frame and a large non-functional fusion protein (38), the second report the same year (41) showed that it actually results in a greatly truncated form of the enzyme. Comparisons with our closest living evolutionary relatives (42) indicated that this mutation occurred after our common ancestry with these "great apes" (**Figure 1**).

Possible Selection Mechanisms for the Initial Hominin Mutation in *CMAH*

Whether this mutation got fixed in the human lineage as a result of positive or negative selection is still a matter of speculation. A pandemic caused by a lethal infectious pathogen that preferred to bind to Neu5Gc leading to negative selection is one possible explanation (43). Another mutually non-exclusive possibility is selective fertility of Neu5Gc-deficient females with Neu5Gc-deficient males, leading to positive selection of this genotype (44). This so-called "cryptic female choice" theory (44) is pictorially depicted in **Figure 2** (The figure legend details this theory) (45).

This mechanism was demonstrated in human-like *Cmah* null mice (44, 46). On the other hand, a random *CMAH* mutation may simply have become fixed in a small group of individuals who eventually gave rise to modern humans.

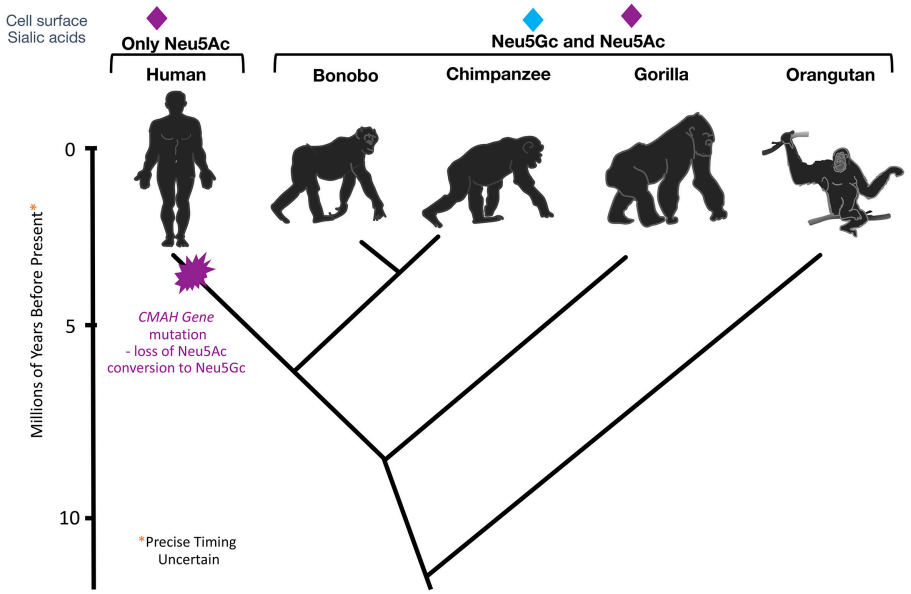

FIGURE 1 | Evolutionary Loss of *CMAH*. Multiple methods of analysis indicate that the *CMAH* mutation occurred about 2–3 mya after the divergence from the Pan group.

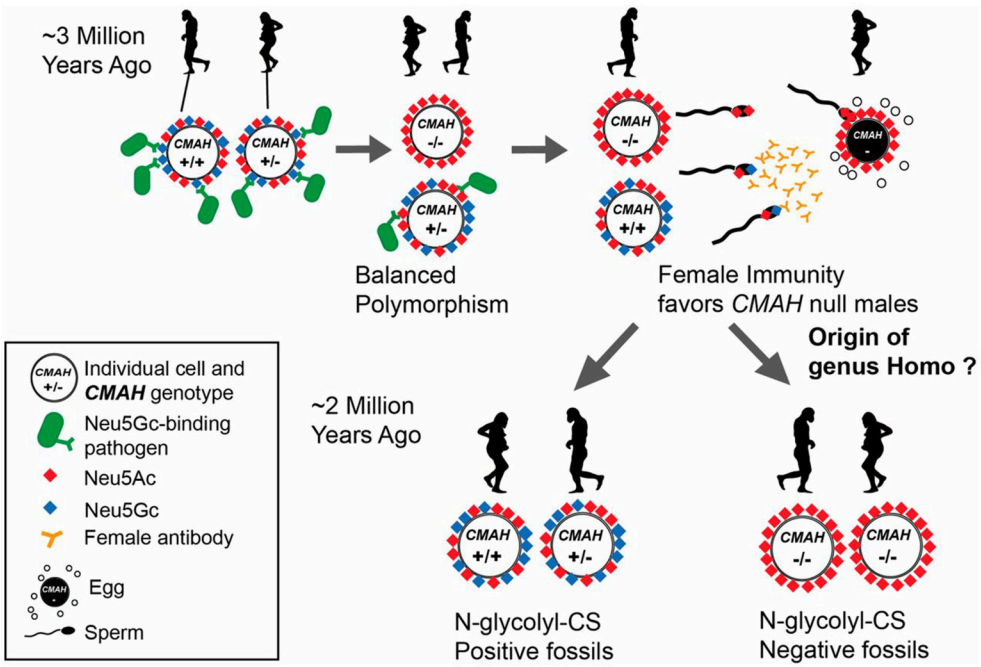

FIGURE 2 | Potential scenario for the role of Neu5Gc loss and female anti-Neu5Gc immunity in the origin of the genus *Homo* via interplay of natural and sexual selection acting on cell-surface Sias. There are many known pathogens that recognize and exploit Neu5Gc (blue diamond) as a receptor on host target cells. Natural selection by such pathogens may have selected for rare *CMAH* null alleles that abolish Neu5Gc expression in homozygote individuals. Such individuals have only Neu5Ac and its derivatives on their cells (red diamonds) allowing an escape from pathogens, but at higher frequencies would be targeted by adapting pathogens, resulting in maintenance of a balanced polymorphism. $CMAH^{-/-}$ females with anti-Neu5Gc antibodies also present in their reproductive tract would favor sperm from $CMAH^{-/-}$ males due to anti-Neu5Gc antibody-mediated cryptic selection against $CMAH^{+/-}$ or $CMAH^{+/+}$ males expressing Neu5Gc on their sperm. Once the frequency of the *CMAH* null allele reaches a critical level, this process can drive fixation of the loss-of-function allele in a population by directional selection. Figure and figure legend reproduced from Bergfeld et al. (45).

Regardless, this inactivation of *CMAH* lead to drastic changes in the sialoglycome that likely pre-dated the origin of the genus *Homo* (44). Given that Neu5Gc has been found in multiple species of the deuterostome lineage ranging from sea urchins to non-human primates, *CMAH* is at least 500 million years old (47). Interestingly, Neu5Gc was independently lost in multiple lineages including sauropsids (birds and reptiles), monotremes (platypus) and certain other lineages (47, 48). More details about the evolutionary implications of Neu5Gc and anti-Neu5Gc glycan antibodies have been covered by P. Gagneux in another review in this special issue.

HUMANS EXPRESS DIETARY-DERIVED Neu5Gc ON THEIR CELL SURFACES

Neu5Gc-Glycans Are Present in Smaller Amounts in Normal Human Epithelia and Endothelia

Apart from onco-fetal human tissue, very small amounts of Neu5Gc-glycans were surprisingly also found to be incorporated in normal human secretory epithelia and small and large vessel endothelia (36, 37, 49) (**Figure 3**). Concurrent mass-spectrometric studies of purified sialic acids confirmed the presence of Neu5Gc (49) and in *N*-glycans released from tumor samples (50).

Neu5Gc-Glycans in *CMAH* Null Humans and Mice Are Exclusively Derived From Food Sources

Although human cells cultured in FCS have been reported to express Neu5Gc-glycans (42, 51) this appears to be due to metabolic incorporation or passive adsorption of glycoconjugates. So far it seems that the only source of exogenous Neu5Gc in human and humanized *Cmah* null mice is via dietary intake (49, 50, 52, 53) Sialic acids have never been detected in plants and are found in large amounts primarily in vertebrates and a few "higher" invertebrates as well as in some insects (54–58). The occurrence of Neu5Gc in poultry and fish is rare but common in some milk products and greatly enriched in red meats (49, 53, 59, 60).

Red Meat as the Primary Dietary Source of Neu5Gc–The First Example of a "Xenoautoantigen"

With no other explanation for the presence of Neu5Gc-glycans in human tissues as confirmed in the mouse model, it was concluded that humans incorporate Neu5Gc from dietary sources. Studies using a DMB-HPLC assay to detect Neu5Gc showed its enrichment in beef, pork and lamb (53). Additionally, all humans produce anti-Neu5Gc glycan antibodies in varying titers (61). In light of these antibodies that likely bind to any incorporated Neu5Gc-glycans, this is the first example of a "xenoautoantigen." This state, with both the presence of Neu5Gc-glycans as well as the corresponding anti-Neu5Gc glycan antibodies has been called "Xenosialitis" and likely plays a role in multiple human pathologies, as elaborated in later sections of this review.

Mechanisms of Neu5Gc Uptake and Incorporation Into Human Tissues and Cells

When human volunteers ingested free Neu5Gc, it was shown to be largely excreted in the urine (49). Extended feeding of *Cmah* null mice with free Neu5Gc in drinking water also did not result in efficient tissue incorporation except in a malignant tumor (52). In contrast, feeding of glycosidically-bound Neu5Gc attached to porcine mucins gave low-level incorporation into normal tissues over a period of weeks (62). While it has been previously shown that *N*-glycolylmannosamine a degradation product of Neu5Gc which may more easily be taken up than the parental sialic acid (63), the exact mechanism by which bound Neu5Gc from the diet results in metabolic incorporation is not known and requires further investigation.

In contrast, human epithelial cells in culture can metabolically incorporate free or bound Neu5Gc and express it into endogenous glycoproteins (64) (**Figure 4**). The mechanism of uptake and incorporation of the Neu5Gc into human epithelial cells (derived from a primary colon carcinoma), fibroblast, and neuroblastoma cells was shown to be dependent on non-clathrin-mediated pinocytic pathways (64). Free Neu5Gc taken up by pinocytosis, or bound Neu5Gc released by a lysosomal sialidase, can then be exported to the cytosol by the lysosomal sialic acid transporter. Activation of the resulting cytosolic free Neu5Gc by the CMP-sialic acid synthase then generates the donor for incorporation into glycoconjugates in the Golgi apparatus, on newly synthesized glcoconjugates. The reason why free Neu5Gc gives incorporation in cultured cells but not in the intact organism is because of the rapid clearance by the kidney in the latter situation. The difference between free and bound Neu5Gc is also relevant to recognition by antibodies which can only interact with the latter. Moreover, the typical antibody binding site can accommodate glycan chains of 4–6 monosacharride (66). Antibodies typically cannot efficiently recognize just a terminal Neu5Gc even when glycosidically bound. For this reason, many studies that have utilized simple alpha-linked Neu5Gc as a target in ELISA assays grossly underestimate the amount and complexity of anti-Neu5Gc glycan antibody response (67). Hereafter, we therefore refer to antibodies against glycosidically-bound Neu5Gc as "anti-Neu5Gc-glycan antibodies" which are diverse and complex because of the underlying glycans.

Metabolic Fate of Neu5Gc

As the reaction catalyzed by Cmah is irreversible, all mammalian cells must have pathways to adjust cellular Neu5Gc levels to their needs to avoid continued accumulation. We discovered a metabolic pathway for the turnover of exogenous Neu5Gc in human cells (68). It was shown that cytosolic extracts harbor the enzymatic machinery to sequentially convert Neu5Gc into *N*-glycolylmannosamine, *N*-glycolylglucosamine, and

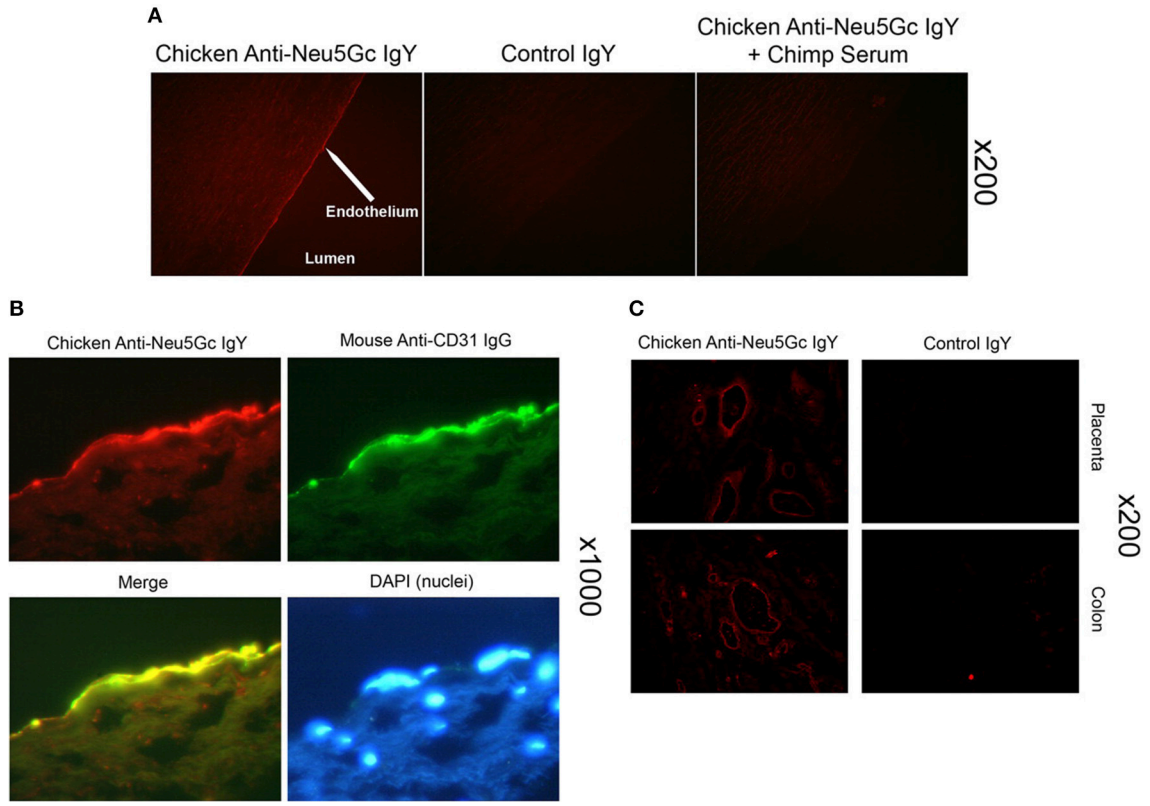

FIGURE 3 | Detection of Neu5Gc in aortic endothelium of human autopsy samples and microvasculature of colon and placenta. The chicken anti-Neu5Gc antibody (cGcAb) was used to detect the presence of Neu5Gc on the endothelium of autopsy samples of normal-appearing human aorta. Typical representatives of 8 autopsy samples studied are shown. The red Cy3 fluorescence represents labeling of endothelial cells of the aorta. **(A)** Specificity of the antibody was demonstrated by the lack of signal with the non-immunized control chicken IgY (middle) and the abrogation of signal by adsorption with Neu5Gc-rich glycoproteins of chimpanzee serum (right). Magnification ×200. **(B)** Sections were double-stained with anti-CD31 for endothelial cells and counterstained with DAPI to visualize nuclei (magnification ×1000). **(C)** Sections of placenta (top) and colon (bottom) stain for Neu5Gc along microvasculature endothelial lining with the use of cGcAb. Control IgY (right) demonstrates specificity of signal (magnification ×200). Figure and figure legend reproduced from Pham et al. (37).

N-glycolylglucosamine 6-phosphate, whereupon irreversible de-*N*-glycolylation of the latter results in the ubiquitous metabolites glycolate, and glucosamine 6-phosphate. Later, it was shown that metabolic turnover of the dietary Neu5Gc in humans and *Cmah* null mice modifies chondroitin sulfate and this stable *N*-Glycolyl chondroitin sulfate (Gc-CS) survives even in ancient fossils (45). This discovery opened a door for "ancient glycomics" and could help in tracking early human lineages and their food habits. Additionally, we are working on developing a simplified assay to measure levels of Gc-CS in serum to predict red meat-related incorporation.

Parallel studies of the *P. falciparum* malarial protein VAR2CSA that mediates parasite attachment to the placental trophoblast led to discovery of the target "oncofetal chondroitin sulfate" (ofCS) which is not detected in normal tissues, but is shared by many types of cancers and can be detected using recombinant VAR2CSA(rVAR2) (69–72). As this pattern is similar to that of Neu5Gc-glycans in placental and tumor tissue, it was natural to suspect that it might be related to Gc-CS. However, this matter requires further investigation.

HUMANS ALSO HAVE ANTI-Neu5Gc ANTIBODIES

All Humans Have Circulating Anti-Neu5Gc-Glycan Antibodies

All human adults have varying levels of circulating IgM, IgG, and IgA antibodies against Neu5Gc-glycans (49, 61, 73–75). Human anti-Neu5Gc glycan antibodies interact with metabolically incorporated Neu5Gc to promote chronic inflammation, likely contributing to tumor inflammation and cancer progression (50, 53) and vascular inflammation (37).

Origin of Human Anti-Neu5Gc-Glycan Antibodies

Our group later showed that human anti-Neu5Gc glycan antibodies appear during the first year of life and correlate with the introduction of Neu5Gc in the diet (76). Sera from infants aged 0–12 months were analyzed, and anti-Neu5Gc IgM and IgG antibodies against Neu5Gcα2-6Lac started to appear at the time these infants were weaned on to cow's milk-based formula. Interestingly, anti-Neu5Gc IgM antibodies were absent

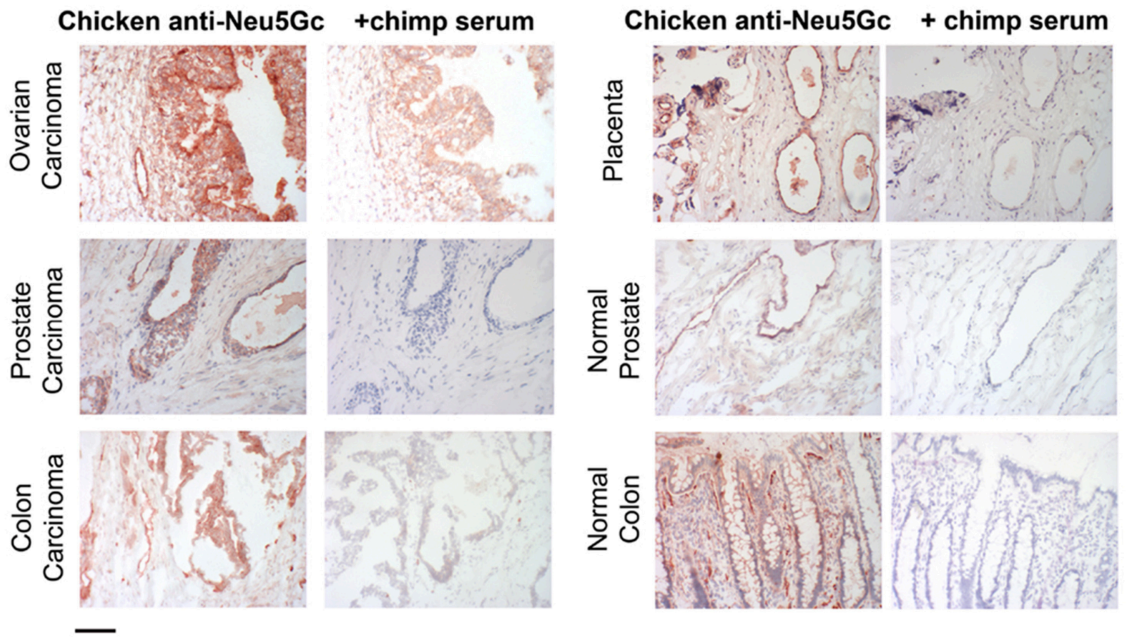

FIGURE 4 | Examples of incorporation of Neu5Gc in malignant and healthy human tissue. Expression of Neu5Gc is observed to be enhanced in malignant epithelia as seen here in carcinomas of the ovary, prostate and colon (left panel). In contrast, expression of Neu5Gc in normal tissue is seen in the ducts of the prostate gland and in the epithelial lining of the colon (Right panel). Endothelial cells of the normal placenta are used here as a positive control for Neu5Gc immunostaining. As a negative control, the binding is blocked competitively with Neu5Gc-containing chimpanzee serum. Magnification used was 200× and scale bar is 100 μm. Figure and figure legend reproduced from Samraj et al. (65).

at birth and at 3 months, appeared at 6 months and the levels stabilized at 12 months. There was no difference in anti-Neu5Gc IgM and IgG titers between male and female subjects. The absence of anti-Neu5Gc IgM antibodies in cord blood sera suggests that anti-Neu5Gc antibodies are not germ-line encoded "natural" antibodies (77) that occur naturally in human and other mammals, but instead require a postnatal antigenic stimulus. Anti-Neu5Gc antibodies are likely to be affinity matured antibodies as has been shown earlier (78). However, spontaneous generation of anti-Neu5Gc IgM or IgG antibodies in *Cmah* null mice did not occur even when large quantities of Neu5Gc were fed to them. This is despite the presence of relatively hyper-reactive B cells, apparently caused by the loss of Neu5Gc-containing Siglec ligands (79, 80). On the other hand, deliberate immunization with an artificial immunogen rich in Neu5Gc, such as chimpanzee RBCs, and complete Freund's adjuvant, did elicit anti-Neu5Gc IgM, and IgG antibodies in *Cmah* null, but not in wild type mice (50, 75).

N-Glycolyl Groups Are Rare in Nature, Increasing the Likelihood of Antigenicity

N-acetyl groups are common in nature (PubMed search of "*N*-Acetyl" gives >30,000 citations), often originating from the donor acetyl-CoA. In contrast, a search of "*N*-Glycolyl" gives ∼270 citations, which are either about Neu5Gc or about *N*-Glycolylmuramic acid, found in certain bacterial peptidoglycans (81–86). The *CMAH* gene is a distant homolog of prokaryotic genes generating UDP-*N*-glycolylmuramic for peptidoglycan biosynthesis (82, 83). In both instances, a mono-oxygenase reaction is involved. It is unclear why glycolyl-CoA formed during fatty acid beta-oxidation (87, 88) is never utilized to make *N*-glycolyl groups. Regardless, the rarity of this modification makes it more likely to be antigenic. *N*-glycolylmuramic acid occurs in Freund's adjuvant (which has mycobacterial products), which we use to immunize *Cmah* null mice against Neu5Gc-glycans, but we do not observe anti-Neu5Gc Abs in mice given only adjuvant.

Markedly Different Antigenicity of Glycosidically-Bound vs. Free Neu5Gc and Impact of Underlying Glycans

As was touched upon earlier, the difference between free and bound Neu5Gc is also relevant to Ab recognition, which can only interact with the latter. Moreover, since the typical Ab binding site accommodates 4 to 6 monosaccharides (66, 89, 90), Neu5Gc-dependent Abs cannot efficiently recognize a terminal glycosidically-bound Neu5Gc by itself. Thus, studies that utilized simple alpha-linked Neu5Gc as a target in assays (67, 91–95) grossly underestimate the complexity of the human anti-Neu5Gc Abs, which are diverse and complex, because of variations in underlying glycans (61, 96, 97). Recently, it has also been shown that the presentation mode of Neu5Gc-containing glycans in various assays affects recognition by anti-Neu5Gc glycan IgGs (98).

Possible Mechanism of "Xenoauto-Immunization" by Microbes Like *Haemophilus influenzae*

While humans develop antibodies against Neu5Gc-containing glycans during infancy, the mechanism of immunization is still unclear. One possible explanation is "xeno-autoimmunization" by microbes such as *H. influenzae,* that normally colonize humans. Non-typeable *H. influenzae* (NTHi) like all other known microbes cannot synthesize Neu5Gc but has been shown to be able to incorporate trace amounts of free sialic acids into its cell-wall LPS (99). Also, anti-Neu5Gc antibodies appear in infants around the same time as antibodies against NTHi (76). One likely source of Neu5Gc for these microbes is foods of mammalian origin used for weaning. Indeed, NTHi was shown to be able to incorporate Neu5Gc from baby foods (76).

A Parallel but Inconsistent Literature About Anti-tumor MAbs Against (Neu5Gc)GM3

An extensive literature originating primarily from one group (100–113) claims that a Neu5Gc-version of ganglioside GM3 (Siaα2-3Galβ1-4Glcβ1-1'-Ceramide) is tumor-specific, and cancer vaccines and MAbs (idiotypic and anti-idiotypic) targeted against it are even in clinical trials (114, 115). Until recently this group assumed that expression was unrelated to dietary intake, and that the antigen is absent from normal cells. Moreover, a collaborating group recently suggested that hypoxia induces *de novo* synthesis of (Neu5Gc)GM3 in human cells through a poorly defined "*CMAH* domain substitute" (116). However, hypoxia also increases uptake and incorporation of Neu5Gc, and fetal calf serum contains Neu5Gc. Once human cancer cells are placed in Neu5Gc-free human serum, for several passages, we find that all traces of Neu5Gc disappear. Moreover, our broad-spectrum polyclonal monospecific chicken anti-Neu5Gc Ab cannot detect any Neu5Gc in *Cmah* null mice on a Neu5Gc-free diet. Further confusion arises because the original group also uses these antibodies to treat tumors in *Cmah* wild type mice (107), which already have a large amount of endogenously synthesized Neu5Gc-GM3. This is also true of preclinical toxicity studies done in *CMAH*-positive monkeys (117). We may be misunderstanding something about this body of work, but our present assumption is that the tumor-associated (Neu5Gc)GM3 being targeted arises from dietary Neu5Gc. Alternatively, the actual epitope may be different. Regardless of the final resolution, it does not change the basic underlying hypothesis driving our current work, on red meat-derived Neu5Gc-induced "xenosialitis."

ANTI-Neu5Gc ANTIBODIES IN DISEASE STATES

As alluded to earlier, anti-Neu5Gc antibodies have been described in a multitude of diseases. Anti-Neu5Gc antibodies have broad implications in transplantation (93, 118–125) which will be

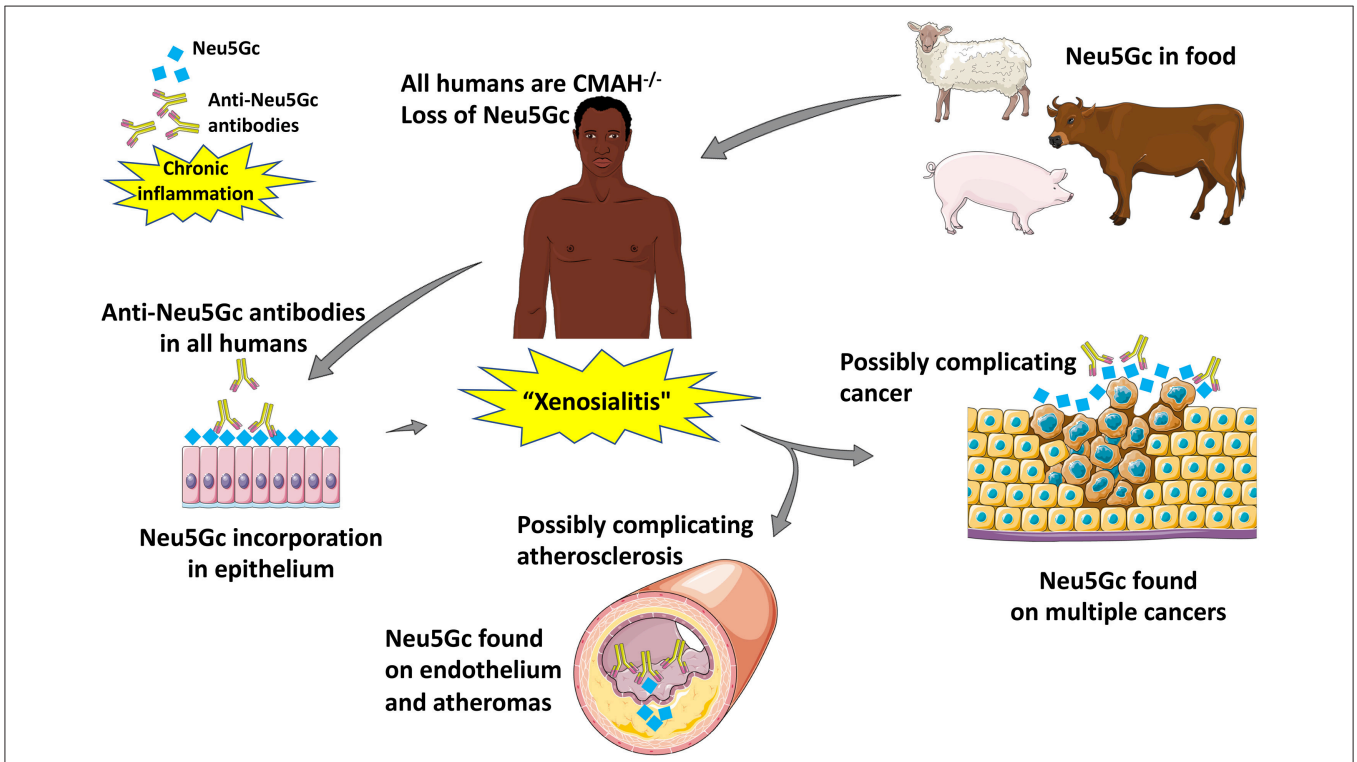

FIGURE 5 | Suggested pathological implications of Neu5Gc consumption, accumulation, and subsequent inflammation in atherosclerosis and cancer [Image created with objects sourced from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Common Attribution 3.0 Generic License].

covered in a separate review in this special issue. While transplantation can be associated with high levels of anti-Neu5Gc-glycan antibodies due to ATG serum therapy and/or the xenotransplant itself, these are very unusual clinical states with associated immunosuppression and other pathologies. Also of note, the phenomenon of "hormesis" has been documented with these antibodies, with very highly levels having the opposite effects e.g., killing of tumors (126, 127). In this review, we will focus on a possible role of moderate levels of the antibodies in two diseases that otherwise normal humans are particularly prone to develop: epithelial cancers (carcinomas) and atherosclerosis (**Figure 5**).

Carcinomas

Accumulation of Neu5Gc-glycans has been detected in human tumors such as breast, colon, ovary, and prostate carcinomas (49, 65, 128, 129). Distinctly, red meat is enriched with bound forms of the Neu5Gc. Numerous epidemiological studies concluded that consumption of red meat is associated with atherosclerotic cardiovascular diseases and an increased risk of cancer (130, 131). Recent findings involving the Health Professionals Follow-up Study and the Nurses' Health Study cohorts confirmed that a higher intake of red meat (specifically processed red meat products) was associated with a significantly elevated risk of cancer, prominently colorectal cancer (132). The epidemiological data ruled out alternate factors such as (a) high-fat intake (133); (b) the production of heterocyclic amines and polycyclic aromatic hydrocarbons (134); (c) the presence of mutagenic *N*-nitroso compounds (135), that were once believed to be the major promoter of carcinogenesis. Our laboratory has shown that the Neu5Gc and anti-Neu5Gc-glycan antibody interaction induced "xenosialitis" may promote chronic inflammation leading to cancer progression (53).

Another possibly related carcinogenic mechanism arising from red meat was revealed by the isolation of a number of small DNAs obviously derived from specific plasmids of Acinetobacter bacteria from commercially available cow milk samples by de Villiers and zur Hausen (136–138). These authors suggest that such infections with autonomously replicating plasmids early in life are risk factors for human colon and breast cancers several decades later (139), that incorporated Neu5Gc from dietary sources might provide receptors for the viruses, and that antibodies against these viral proteins may work in concert with Neu5Gc-induced "xenosialitis."

As has been shown earlier, inflammation and associated activation of the immune system can promote carcinogenesis (inflammation-induced cancer) and cancer progression (140–142). The seminal review on the hallmarks of cancer by Hanahan and Weinberg also mentions tumor-promoting inflammation as one of the enabling factors of cancer (143). Moreover, growing tumors induce an inflammatory response that can support cancer progression (cancer-related inflammation) (140, 144). Chronic inflammation in auto-inflammatory diseases and diet-induced metabolic syndrome is also an important etiological factor for the development of cancer (142, 145). Hence it is not surprising that red meat consumption and the "Western diet" have often been associated with increased circulating markers of inflammation in human population studies (146). Cell surface glycosylation is heavily altered in cancer cells, as seen in malignant tissue that incorporate Neu5Gc (62, 64, 147). Thus, anti-Neu5Gc antibodies likely support cancer progression by enhancing tumor-related inflammation via induction of "xenosialitis" in the humanized mouse model ($Cmah^{-/-}$) (53, 148, 149). A recent study showed that there is no increase in colon cancer risk following anti-Neu5Gc antibody induction with Neu5Gc-bearing rabbit anti-T cell IgG (ATG) in recipients of kidney (150). However, there was no estimation regarding red meat intake in this study and patients with renal failure are typically advised to reduce meat intake. Furthermore, some such patients are also under immunosuppression, which would alter outcomes*.

Sialoglycan microarray studies enabled us to differentiate between controls and patients with various carcinomas including prostate, ovary, endometrium, colon, lung, and pancreas with regard to antibodies against Neu5Gc-Sialyl-Tn (96). A recent nested case-control study from our laboratory assessed the association between total anti-Neu5Gc antibodies and the risk of colorectal cancer (CRC) in the Nurses' Health Study cohort. This study showed that the sum total of polyclonal anti-Neu5Gc glycan antibodies were associated with CRC risk (97).

Atherosclerosis

Myocardial infarctions (MIs), ischemic heart disease, strokes and peripheral vascular disease in humans are primarily caused by atherosclerotic cardiovascular disease (CVD) (151). Chimpanzees, our closest evolutionary cousins, on the other hand suffer from "heart attacks" as a result of idiopathic interstitial myocardial fibrosis (152). Additionally, captive chimps do not get human-like MIs despite major risk factors such as dyslipidemia and hypertension (152). There is a clear association between consumption of red meats and processed meats with increased risk of CVD in humans (131, 153). While multiple theories for this association have been put forward including cholesterol and saturated fat (154), conversion of choline and carnitine into proatherogenic Trimethylamine N-oxide (TMAO) (155–157), and oxidative damage due to heme iron (158–161), these mechanisms appear not to be specific for red meats as explained in an earlier review from our laboratory (162). "Xenosialitis," unlike these theories, is specific to red meats and may contribute to the uniquely human severity of complications of atherosclerosis. Earlier studies from our lab have shown that Neu5Gc can be detected in the endothelium overlying the atherosclerotic plaque as well as the sub-endothelium (37). Further, human endothelial cells fed with Neu5Gc and subsequently exposed to serum containing anti-Neu5Gc glycan antibodies led to IgG and complement deposition which in turn led to increased endothelial activation, increased cytokine production, and selectin expression, events associated with early atherogenesis. These effects were inhibited by Neu5Gc-alpha-methyl glycoside, a specific competitor to anti-Neu5Gc antibodies. $Cmah^{-/-}$ mice also showed Neu5Gc accumulation in their endothelium when fed with Neu5Gc (62). We are currently studying $Cmah^{-/-}$ mice bred into a low-density lipoprotein knockout ($Ldlr^{-/-}$) background fed with Neu5Gc and immunized with Neu5Gc bearing antigens to see if they

have a higher risk of developing atherosclerosis as compared to controls fed Neu5Ac. Large human cohort studies are also necessary to confirm the role of anti-Neu5Gc antibodies in CVD.

CLINICAL APPLICATION OF ANTI-Neu5Gc GLYCAN ANTIBODIES

Possible Therapeutic Role of Neu5Gc-Antigens and Anti-Neu5Gc Antibodies

Despite the possible pathogenic effects of these antibodies as described above, anti-Neu5Gc antibodies may also be potentially utilized as anti-cancer immunotherapeutic agents. Tumor cells are aberrantly sialylated and the content of sialic acid on these cells goes up markedly when compared to cells of healthy tissue (163, 164). This upregulation may explain why ingested Neu5Gc preferentially accumulates in cancer tissue (49, 62). There is also an upregulation of sialyl-Tn antigen (165–169), an epitope not commonly found (165, 170, 171) or "hidden" by *O*-acetylation of sialic acid (166) in healthy human tissue. Recent findings also show the presence of Sialyl-Tn in stem-like cells in cancer cell lines (172) and therapeutic benefits of antibodies that target these epitopes in patient-derived xenograft models of Ovarian carcinoma (173). If Neu5Gc-Sialyl-Tn is found to be relatively cancer specific, it may be used to image or even treat cancers. Indeed, *in vitro* assays have shown that human antibodies against Neu5Gc-Tn antigen purified from IVIG activate antibody-dependent cellular and complement-dependent cytotoxicity (ADCC and CDC) (96).

Another approach that has been tried is vaccination with (Neu5Gc)GM3 along with outer membrane protein complex of *Neisseria meningitidis* in proteoliposomes leading to antibody production in advanced stage breast cancer patients in a phase I study (174). A mouse-monoclonal antibody directed against (Neu5Gc)GM3, 14F7 was isolated (129) and further, has been humanized (175). 1E10, the corresponding anti-idiotype to 14F7, named racotumomab has also been tried in humans (176) and also shown to have non-apoptototic cytoxic effects *in vitro* (177). This antibody is able to bind to multiple malignant tissues including skin cancers, neuroectodermal tumors, genitourinary cancer, non-small cell lung cancer, and gastrointestinal tumors (178–182) and multiple human trials have also been conducted (e.g., NCT01598454, NCT01460472, NCT02998983, NCT01240447). However, as mentioned earlier, these studies do not make any direct link to dietary Neu5Gc, and the antibodies are reported to work even in *Cmah* wild-type mice, which have a vast excess of Neu5Gc antigens on normal tissues.

Despite all these efforts to develop effective immunotherapeutics, no efforts have been taken to control Neu5Gc consumption in cancer patients. Notably, if cancer patients are encouraged to reduce Neu5Gc consumption, a "washout" of Neu5Gc may occur in normal tissue. Following this, IV Neu5Gc may be used to "feed" tumors followed by an antibody that recognizes Neu5Gc-containing epitopes to now "find" the tumor. "Feeding" tumors is possible as Neu5Gc preferentially accumulates in malignant tissue due to increased micropinocytosis (64), rapid growth rates and hypoxic upregulation of the sialin transporter (147). This "feed-and-find" approach may turn out to be more effective than the present approaches. Additionally, monoclonal antibodies targeting Neu5Gc-containing glycans may be tested on an advanced sialoglycan microarray (183) and coupled with a newly developed computational methods (184) to confirm specificity.

Importantly, Neu5Gc has also been found in cancer therapeutic agents. Monoclonal antibodies such as trastuzumab, cetuximab and rituximab are integrated in today's cancer therapies (185). Glycosylation of these antibodies may involve Neu5Gc-rich media and/or mammalian cells that express Neu5Gc (186). Our laboratory has previously shown that incorporation of Neu5Gc in cetuximab enhanced the formation of immune complexes promoting drug clearance (187). Avoidance of Neu5Gc during production of glycoproteins may improve half-life of these antibodies while also reducing their immunogenicity.

Biomarkers in Pathological States

Anti-Neu5Gc glycan antibodies could serve as potential biomarkers for diseases associated with red meat consumption including carcinomas, atherosclerosis, and type 2 diabetes (188–192). Current biomarkers for cancer lack sufficient sensitivity and importantly the specificity for early diagnosis (193, 194). Although antibodies against tumor-associated antigens are commonly found in cancer patients at an early stage and could potentially be sensitive detectors for malignant transformation (195, 196), none of the previously described autoantibodies show sufficient specificity in screening. Given the incorporation and display of Neu5Gc by tumor cells, the detection of Neu5Gc body-burden and antibody response together might serve as a potential biomarker for early carcinoma detection. It has been demonstrated that comparison of anti-Neu5Gc antibody levels can be used to differentiate between controls and patients with various carcinomas (96, 97). Increased anti-Neu5Gc antibody levels were also found in patients with Kawasaki disease (197).

CONCLUSIONS AND PERSPECTIVES

In this review, we have discussed important milestones from the early description of "Serum-sickness" as being due to antibodies directed against Neu5Gc epitopes all the way to the present-day therapeutic implications of these antibodies in cancer therapy. Some of these milestones have been represented in a concise timeline (**Figure 6**). While the "Xenosialitis" hypothesis is well-supported in the human-like mouse models, it has yet to be conclusively proven in humans. It remains to be seen if "Xenosialitis" plays a role in other uniquely-human diseases.

There also remain certain unresolved complexities of food sources of Neu5Gc and their propensity for metabolic incorporation. It is noteworthy that processed red meat is much more closely associated with disease risk than red meat *per se*. This is usually explained on the basis of preservatives added to process red meat. However, the same preservatives are added to other foods but are not associated with the same disease

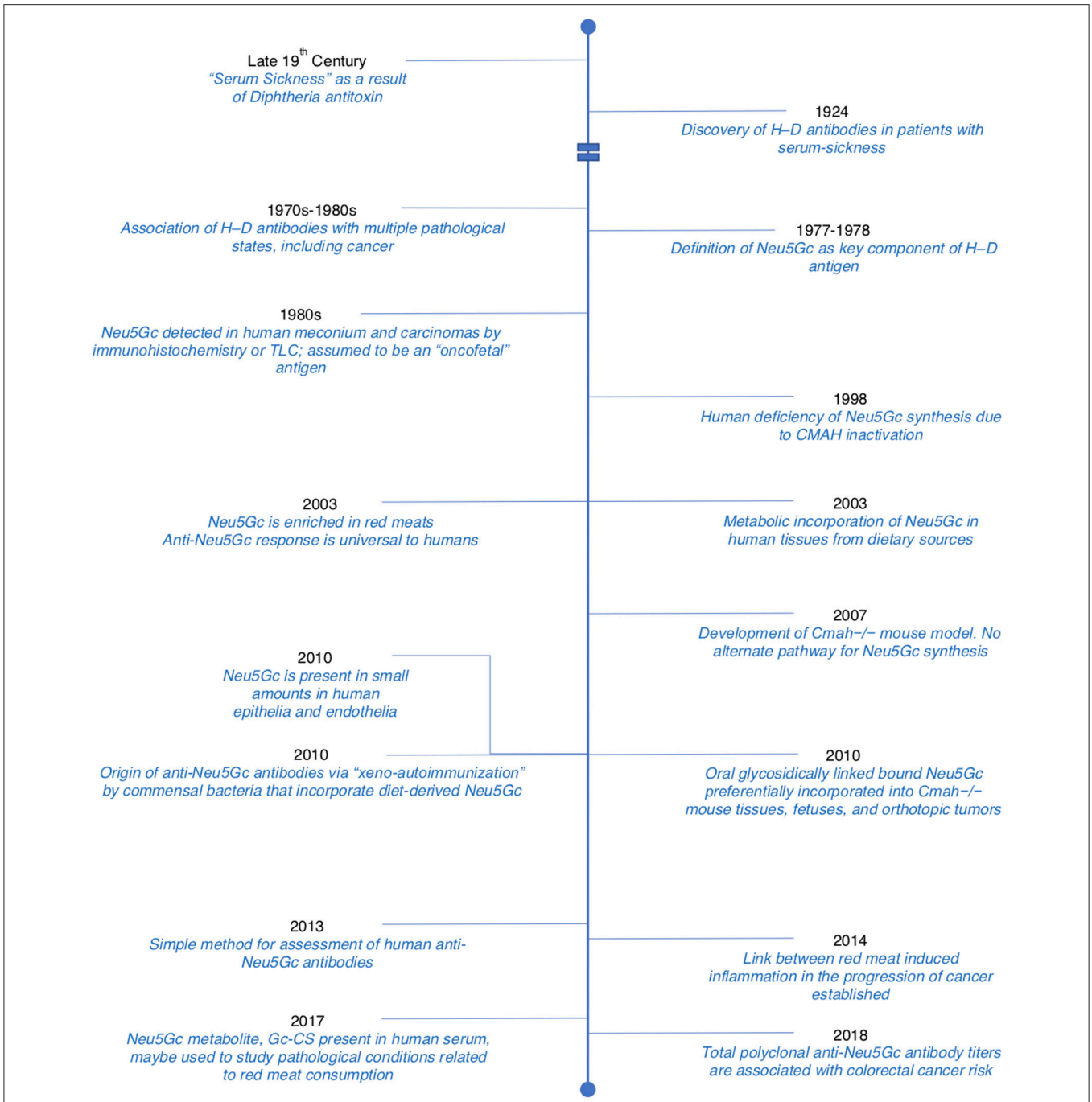

FIGURE 6 | Timeline detailing important discoveries related to Neu5Gc and anti-Neu5Gc antibodies. Adopted and modified from Samraj et al. (65).

risks. One possible explanation is that the predigested nature of the processed food enhances absorption and incorporation of Neu5Gc. In this regard, there is currently no assessment of the relative impact of different foods and food processing on absorption in general. What is needed is that the equivalent of a glycemic index for the impact of glucose uptake (198, 199), i.e., "a GCemic index." Along the same lines we are also missing an equivalent of the HbA1c (198, 199) as an index of long-term metabolic incorporation. We are currently studying the novel metabolite *N*-Glycolyl-chondroitin sulfate as a candidate.

It is also important to emphasize that there are other dietary sources of Neu5Gc besides red meat. While poultry is completely free of Neu5Gc, low levels are found in "fish" (which typically refers to the fish muscle). However, it is well-known that other food sources such as fish eggs, sea urchins, goat milk etc. can be high in Neu5Gc, and antibody development and xenosialitis in

societies that consume large amounts of such foods needs to be studied further. Of course, the presence of bound Neu5Gc does not automatically equate to metabolic incorporation.

One other important perspective from these studies on Neu5Gc and anti-Neu5Gc antibodies is the consumption of red meat. With red meat being the richest source of Neu5Gc, abstaining may be the best way to prevent any "xenosialitis" induced pathologies though this would be largely improbable to sustain in the general population. Another possible way to prevent Neu5Gc uptake is to breed genetically-modified *CMAH* null livestock. Like humans, these animals will be unable to synthesize Neu5Gc and thereby prevent human dietary incorporation. But besides worries about "GMOs," one dangerous implication of rearing such livestock is their increased susceptibility to pathogens that bind Neu5Ac which also likely affect humans. This may be combated by growing GMO modified $CMAH^{-/-}$ "cultured meat" that does not synthesize Neu5Gc under strict aseptic conditions. Other alternatives include competing with an excess of the human sialic acid Neu5Ac.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Absence of Neu5Gc and Presence of Anti-Neu5Gc Antibodies in Humans—An Evolutionary Perspective

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The glycocalyx of human cells differs from that of many other mammals by the lack of the sialic acid N-glycolylneuraminic acid (Neu5Gc) and increased abundance of its precursor N-acetylneuraminic acid (Neu5Ac). Most humans also have circulating antibodies specifically targeting the non-human sialic acid Neu5Gc. Recently, several additional mammalian species have been found to also lack Neu5Gc. In all cases, loss-of-function mutations in the gene encoding the sialic acid-modifying enzyme CMAH are responsible for the drastic change in these species. Unlike other glycan antigens, Neu5Gc apparently cannot be produced by microbes, raising the question about the origin of these antibodies in humans. Dietary exposure and presentation on bacteria coating themselves with Neu5Gc from the diet are distinct possibilities. However, the majority of the non-human species that lack Neu5Gc do not consume diets rich in Neu5Gc, making it unlikely that they will have been immunized against this sialic acid. A notable exception are mustelids (ferrets, martens and their relatives) known for preying on various small mammal species rich in Neu5Gc. No studies exist on levels of anti-Neu5Gc antibodies in non-human species. Evolutionary scenarios for the repeated, independent fixation of *CMAH* loss-of-function mutations at various time points in the past include strong selection by parasites, especially enveloped viruses, stochastic effects of genetic drift, and directional selection via female immunity to paternal Neu5Gc. Convergent evolution of losses of the vertebrate-specific self-glycan Neu5Gc are puzzling and may represent a prominent way in which glycans become agents of evolutionary change in their own right. Such change may include the reconfiguration of innate immune lectins that use self-sialic acids as recognition patterns.

Keywords: sialic acid, Neu5Gc, Neu5Ac, xenoglycan, anti-glycan antibodies, xeno-antigen

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INTRODUCTION

The glycocalyx of all vertebrate cells is decorated with abundant terminal sialic acids. These acidic nine-carbon backbone sugars cap the ends of tens to hundreds of millions of glycan chains per cell. In mammalian species and other vertebrates, the sialic acids *N*-acetylneuraminic acid (Neu5Ac) and its derivative *N*-glycolylneuraminic acid (Neu5Gc) are the two most common forms, each a

family of molecules with various modifications of the canonical, 9-carbon monosaccharide (1). Until recently, humans were the only mammalian species known to lack the sialic acid Neu5Gc, as our lineage fixed the loss-of-function mutation affecting the single copy *CMAH* gene that encodes the sialic acid-modifying enzyme CMAH over 2 million years ago in the lineage leading to *H. sapiens* (2, 3). More recently, several other species of mammals have been documented to also lack Neu5Gc due to ancient mutations fixed over 30 million years ago in these lineages (4–6). The loss of function of the CMAH enzyme prevents the modification of the precursor monosaccharide to the derived sialic acid Neu5Gc (in their respective sugar-nucleotide form, CMP-Neu5Ac and CMP-Neu5Gc). As illustrated in **Figure 1**, the lack of this enzymatic function can lead to drastic changes in the molecular composition of the glycocalyx of cells throughout the body. Recent evidence has shown that humans are not alone in this loss, instead several other species of mammals have independently fixed different loss-of-function mutations of their *Cmah* gene at various time depths during evolution, leading to loss of Neu5Gc in entire lineages or just individual species (6). These losses have occurred through exon deletion, premature stop codons, or frameshift mutations in the gene encoding the CMAH enzyme (4–6). The picture emerging is that of a phylogeny of mammals punctuated with taxa that have lost the capacity to synthesize Neu5Gc (**Figure 2**). These taxa include New World primates (>100 species of South and Central American primates known as *Platyrrhynes*), *Mustelidae* (57 species of small carnivores including ferrets, martens and weasels), pinnipeds (33 species comprising seals, sea-lions and walruses), *Procyonidae* (∼15 species including racoons, ring-tails and coatis,) hedgehogs (17 species), bats from at least two different lineages, sperm whale (a single species), and white-tailed

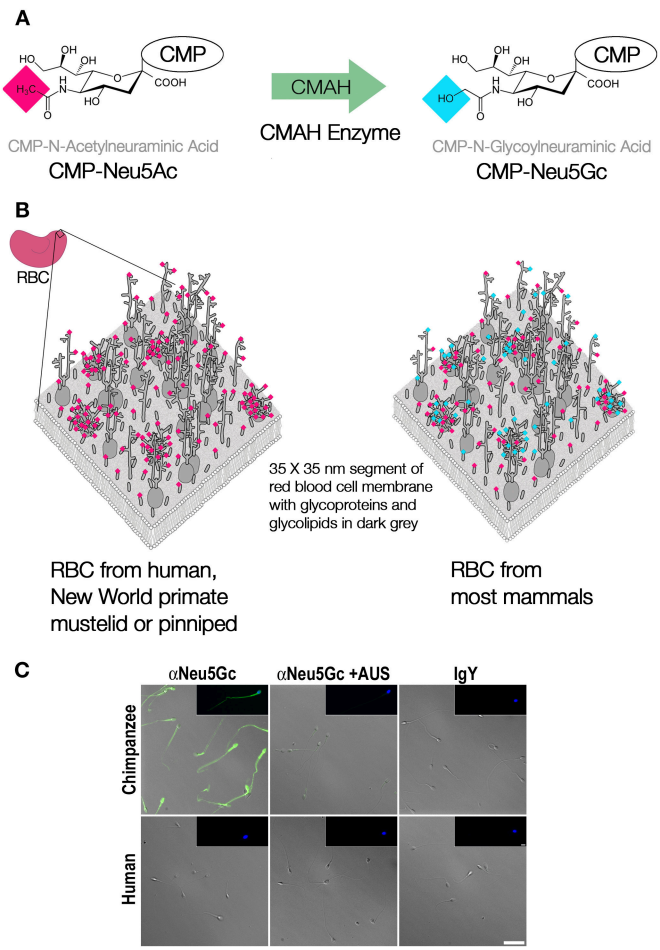

FIGURE 1 | Modification of CMP-Neu5Ac to CMP-Neu5Gc. **(A)** The enzyme CMAH, encoded by a single gene in all mammals, catalyzes the derivatization of Neu5Ac to Neu5Gc in the form of their sugar nucleotides, cytidine monophosphate (CMP). **(B)** Due to the large number of sialic acids terminating many of the glycan chains on the glycocalyces of most cells, the loss of function of the *CMAH* gene leads to a drastic change in the molecular identity or "flavor" of the glycocalyx, as indicated by a small fraction of a red blood cell membrane, redrawn and modified from Viitala and Järnefelt (7). **(C)** Micrographs showing green immunofluorescent staining of Neu5Gc on chimpanzee but not human sperm cells stained with affinity purified chicken anti-Neu5Gc IgY antibody and fluorescent secondary, controls include sialidase treated or anti-IgY secondary antibody alone, nuclei stained by DAPI (blue) reprinted from Ghaderi et al. (8) with permission.

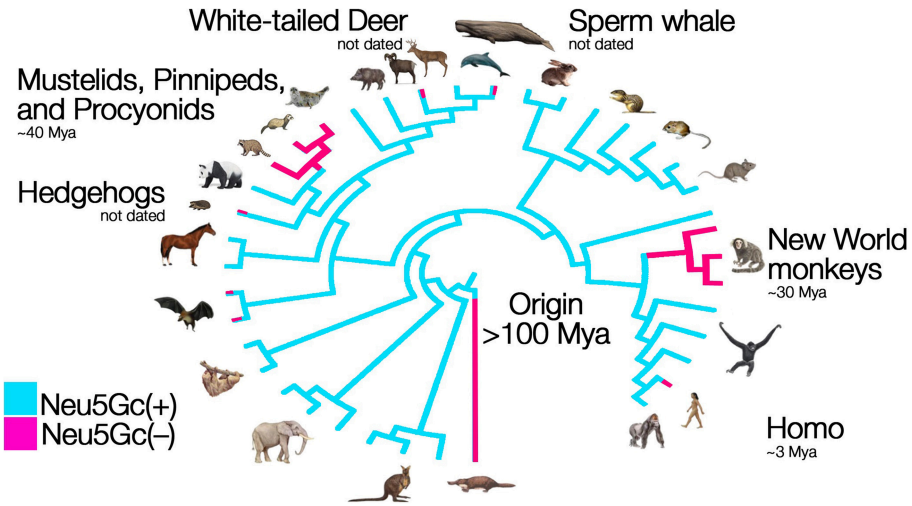

FIGURE 2 | Parallel evolution and loss of an innate self-signal. Humans cannot synthesize Neu5Gc, because human *CMAH* was inactivated over two million years ago (red). The inactivating mutation apparently fixed rapidly after originating, which suggests that the loss could have been adaptive—driven by pathogen avoidance, reproductive conflict, or a combination of the two. Independent losses of Cmah function have recently been found in New World Primates, Mustelids and several other groups. Figure modified from Springer and Gagneux (9). In some lineages, such as bats and toothed whales, only certain species lost the capacity to make Neu5Gc (indicated by lines that are both blue and red).

deer (a single species) (4–6). For most of these groups, only a few representative taxa and a few individuals have been studied at the genomic level, and so there is the possibility that the *Cmah* gene remained intact or polymorphic in some of the taxa.

An obvious prediction is that additional taxa with inactivated *Cmah* genes will be discovered as additional complete genome sequences are obtained. These cases of convergent molecular evolution result in an overall reconfiguration of the outermost layer of the glycocalyx, now lacking Neu5Gc and carrying an excess of Neu5Ac, given that human and non-human cells retain comparable levels of sialic acid (10) (see **Figure 1B** for red blood cells and **Figure 1C** for sperm cells). Among the many functions of the glycocalyx, molecular identity is paramount (11–13). The molecular patterns, as defined by composition and structure of the glycocalyx have evolved into self-associated molecular patterns (SAMPs) (14), that contribute to efficient surveillance by innate immune receptors including complement factor H and Siglecs, which can inhibit immune response upon engagement with SAMPs (14, 15). Losing Neu5Gc would dramatically alter self-recognition. This would have required evolving altered receptor specificities, affinities, and knock-on effects in signaling pathways due to altered engagement of innate receptors. The biochemical impact of the altered sialome on the human glycocalyx could have had many other effects, including changes in inflammation and metabolism (16, 17).

Another potential consequence are autoreactive antibodies produced against the lost sialic acid. Indeed, despite the absence of endogenous Neu5Gc, experimental studies in humans and in *Cmah*$^{(-/-)}$ mice have revealed that dietary Neu5Gc, in both free and glycoconjugate-bound forms, can become incorporated into tissues in trace amounts. This incorporation occurs especially in tissues with rapid growth and/or turnover rates, including epithelia, endothelia, fetal tissues, and carcinomas (18–20). It has also been established that all humans have various levels of circulating antibodies specific for glycans carrying this foreign molecule, essentially making Neu5Gc a "xeno-autoantigen," which can cause "xenosialitis," an inflammation due to reaction against a xeno-sialic acid that is now part of "self" molecules (21–24). Surprisingly, even humans on diets extremely rich in Neu5Gc do not appear to accumulate beyond trace levels of this dietary xenoglycan.

How ingested Neu5Gc becomes incorporated into the human body remains incompletely understood. There is evidence that Neu5Gc is converted to GalNGc and can then be incorporated into the glycosaminoglycan chondroitin sulfate, an important component of extra-cellular matrices and skeletal bone (25). This incorporation has recently allowed the identification of GalNGc in bones and in fossilized bones as old as 3 million years, opening the possibility to study ancient glycomes of extinct hominins (26). There is much ongoing research to understand the potential effects of incorporation of dietary xeno-sialic acid and targeting antibodies against xeno-sialic acid, xenosialitis in the context of cancer and autoimmunity and even unexplained infertility, where chronic immune reactions to incorporated xenoglycans could contribute to xenosialitis (22–24, 27–29). Aside from humans, natural levels of anti-Neu5Gc antibodies in other species lacking Neu5Gc have not been studied to date. However, anti-Neu5Gc antibodies have been seen in chickens, where antibodies can be efficiently generated upon immunization (18) and are the basis of immune assays for the detection of Neu5Gc in human samples (30).

NATURAL IMMUNIZATION AGAINST NEU5GC-GLYCANS

There are four main differences between immunization against Neu5Gc and other xenoglycans, such as the disaccharide alpha-Gal, or alloglycans such as ABO oligosaccharide antigens. First, in the case of other xenoglycans, immunization against the missing, terminal "self"-glycan is thought to be caused by encounters with microbial glycans with the same structure (31, 32). Considering that the synthesis of endogenous Neu5Gc has never been documented for any microbe, it would appear unlikely that this microbial priming method occurs for Neu5Gc (33, 34). Despite the apparent lack of Neu5Gc synthetic capacity in microbes, however, at least one microbe, Non-typeable *Haemophilus influenzae* (NTHi), can scavenge dietary Neu5Gc and incorporate it into its own glycolipids. There is evidence that young human infants are "xeno-autoimmunized" against Neu5Gc by early *H. influenzae* infection and this method has also been utilized for experimental immunization of $Cmah^{(-/-)}$ mice in the laboratory (35). Immunization thus seems to depend on diets rich in Neu5Gc from red meats or certain marine sources (fish eggs or echinoderms (27, 36). Secondly, unlike other xenoglycans, it is important to stress that the monosaccharide Neu5Gc itself is immunogenic, none of the constituent monosaccharides of alpha-Gal (galactose) or ABO antigens (fucose, galactose, N-Acetylgalactosamine, and N-Acetylglucosamine) are foreign to individuals lacking these structures and once ingested, they are incorporated as non-antigenic glycans or metabolized (37). The antigenicity of other xenoglycans, is largely determined by glycosidic linkages, rather than by the nature of the monosaccharide: galactose-alpha-1,3-galactose for alpha-Gal; fucose-alpha-1,2-galactose for the H antigen; H antigen with N-Acetylgalactosamine for A antigen; or H antigen with alpha-1,3-galactose for B antigen. Thirdly, unlike the other immunogenic glycans, Neu5Gc can be part of numerous different antigens depending on the identity of the sialoglycoconjugate they occur on. Finally, sialic acids are one to several orders of magnitude more abundant than either alpha-Gal or ABO glycans (38, 39). These three differences: dietary origin, antigenicity of the monomer itself, and ubiquity/abundance on the cell surface make Neu5Gc a unique antigen, whose loss may lead to wide-ranging physiological effects (37, 38).

While humans have many dietary sources for Neu5Gc, among the New World primates, there are very few species that eat vertebrate meat. Capuchin monkeys (genera *Cebus* and *Sapajou*) are known to prey on young coati (40), relatives of racoons belonging to the family of *Procyonidae*, and on lizards or birds, but these prey species all lack endogenous Neu5Gc (5, 41). It is thus very unlikely that these New World primates are immunized against Neu5Gc in the wild, but captive capuchin monkeys may be exposed to Neu5Gc through monkey chow containing red meat (Primate Info Net, University of Wisconsin). Hedgehogs and other insectivores, consume mostly insect prey that lack sialic acids and thus can be safely expected not to be naturally immunized against Neu5Gc (6). The same can be said for the different bat species that lack Neu5Gc, as these all feed on insects, fruit, or nectar (42). Pinnipeds (seals, sea-lions and walruses) are all strict carnivores and some of their prey include fish and marine invertebrates that could contain Neu5Gc (43). Studies of pinniped immune responses to sialic acids are urgently needed. The one species of whale also lacking Neu5Gc is the sperm whale (*Physeter catodon*) (6), whose diet consists mostly of giant squid and other cephalopods (squid and octopus) with occasional fish (44). Again, such a diet is unlikely to expose sperm whales to large amounts of Neu5Gc (45), leading to the prediction that they will not have circulating antibodies against the xenoglycan. Mustelids are the one group of species for which it can be assumed that dietary exposure and immunization occurs, as they are all known to feed on a variety of small mammals and vertebrates (46).

EVOLUTIONARY MECHANISMS FOR THE FIXATION OF LOSS-OF-FUNCTION MUTATION

The loss-of-function mutations of the *Cmah* gene are by definition recessive, as one copy of the functional gene suffices to generate a Neu5Gc positive phenotype in a diploid organism.

Balancing Selection Maintaining Polymorphisms

Some animals, including several dog and cat breeds, are polymorphic for Neu5Gc expression. While overall tissue expression is thought to be low, expression on blood cells in these animals can be high (47, 48). Polymorphisms involving Neu5Gc on the ganglioside GD3 exist in felids and are called AB blood groups in domestic cats (not related to primate ABO blood groups), where cats lacking Neu5Gc-GD3 have circulating antibodies specific for Neu5Gc (47). Dog breeds also differ in their expression of Neu5Gc on red blood cell glycolipids (48).

Due to the recessive nature of loss of function mutations, their increase in frequency within a population must be mediated by selection on homozygous carriers, who have fitness advantages conferring higher survival and/or reproductive success. For example, selection for polymorphisms involving a loss-of-function mutation could be based on the accompanying ablation of the glycan used as a receptor by pathogens (49–52).

Examples of Neu5Gc specific pathogens abound including the protozoan malaria parasite *P. reichenowi* (53), the swine pathogen *E. coli K99* (54) and the macaque monkey virus *SV40* (55).

In contrast a number of human-specific pathogens evolved specificity for Neu5Ac, including the causative agent of human malignant malaria *P. falciparum*,the toxins of cholera agent *V. cholerae* (56) and typhoid fever agent *S. typhi* (57), and most influenza A viruses (58). Loss-of-function mutations, especially in polymorphic populations, could also provide partial protection from enveloped viruses that bear the antigenic glycan acquired from the cell membrane of the previous, Neu5Gc positive host. The latter mechanism would be analogous to such protection in alpha-Gal negative Old World primates (59–61) and across ABO mismatched humans (62–65). Such protective mechanisms are thus observed both, between species and within species with existing (balanced)

polymorphisms. Balanced polymorphisms are maintained by frequency-dependent selection, i.e., selection favoring the rare variants, thus preventing their extinction but also preventing their fixation (see **Figure 3**) (66). Such dynamic co-evolutionary processes between pathogens and their hosts are the inspiration behind the terms evolutionary arms race and Red-Queen effects (15, 67).

Fixation of the loss-of-function allele, on the other hand, could happen either via directional selection or genetic drift, where small founder populations consist mostly of homozygous carriers of the loss-of-function mutation. First defined using plants in 1962 (68) and recently applied to primates by Galili (65), the idea of "catastrophic selection" combines these ideas with very strong selection. It is not clear how such "catastrophic selection" differs from short episodes of strong selection, possibly accompanied by demographic bottlenecks, which could also result in the fixation of loss-of-function mutations. Alternatively, selective pressure for *Cmah* loss-of-function could occur through reproductive conflict as discussed below.

Female Immune-Mediated Selection Against Paternal Neu5Gc

Mammalian sperm are highly sialylated as a mechanism to enhance sperm survival and function along the perilous journey through the female reproductive tract to the site of fertilization in the oviduct (69–71). Mammals make anti-sperm antibodies when directly exposed to sperm (72). Major human sperm antigens include, highly sialylated GPI-anchored glycoproteins such as CD52 (73, 74), which in males that have a functional *CMAH*

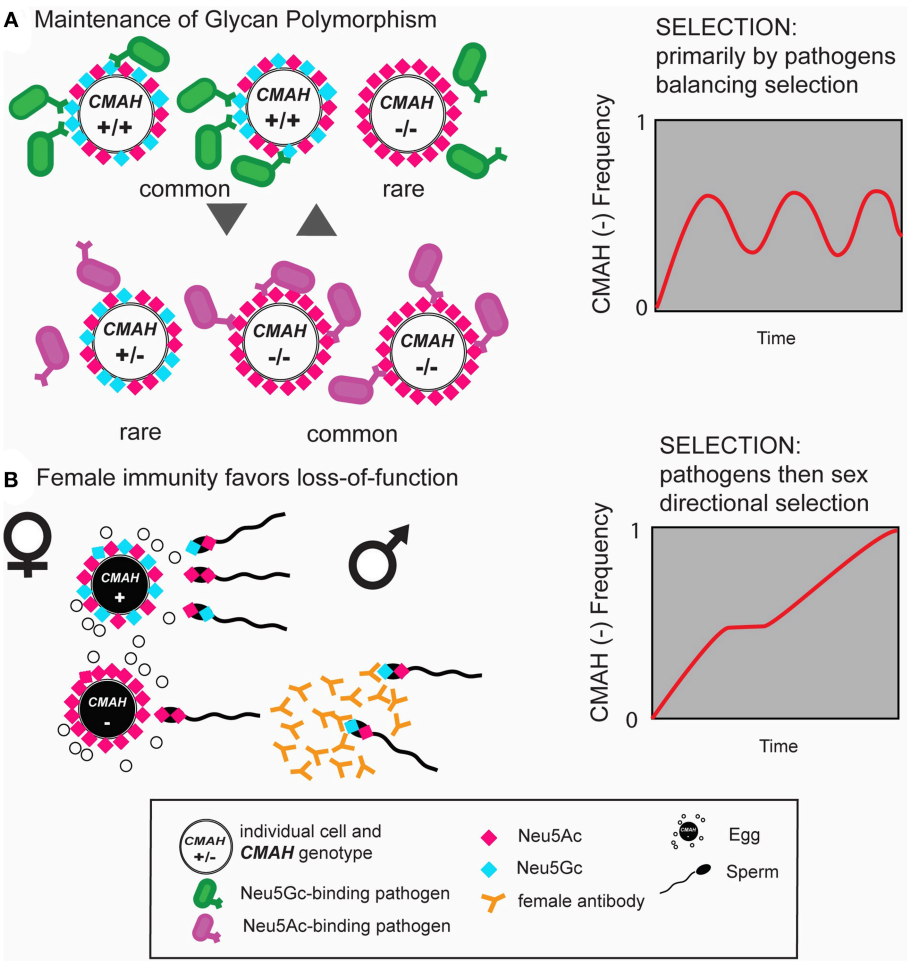

FIGURE 3 | Schematic of the interplay of natural and sexual selection acting on cell-surface sialic acids. **(A)** Natural selection by pathogens recognizing and exploiting Neu5Gc (blue diamond) as a receptor on host target cells can select for mutant *CMAH*$^{(-)}$ alleles that abolish expression of Neu5Gc in homozygote individuals and prevent infection. Such homozygous null individuals have only Neu5Ac on their cells (red diamonds) and at higher frequencies would be targeted by other pathogens adapted or adapting to the host glycan change (magenta). This equilibrium would result in maintenance of glycan polymorphism by balancing selection. **(B)** Anti-Neu5Gc antibody-expressing *CMAH*$^{(-/-)}$ females, immunized by dietary consumption of Neu5Gc rich food (red meat) or by sperm antigens containing Neu5Gc, favor loss-of-function alleles on sperm due to reproductive incompatibility with *CMAH*$^{(-/+)}$ or *CMAH*$^{(+/+)}$ males expressing Neu5Gc on their sperm. Once the frequency of the *CMAH*$^{(-)}$ allele reaches a certain level, this process can drive the fixation of the *CMAH*$^{(-)}$ allele in a population via directional selection. Figure modified from Ghaderi et al. (8).

allele, carry Neu5Gc (75). Theoretically, immunization of females homozygous for the loss-of-function allele of *Cmah* could occur via insemination by males that have Neu5Gc-bearing sperm. Indeed, we have shown experimentally, using $Cmah^{(-/-)}$ mice immunized against Neu5Gc, that their immune response against Neu5Gc bearing sperm severely reduces female fertility (8). In a further study, we demonstrated that Neu5Gc bearing sperm, both, sperm from either wild type mice or from $Cmah^{(-/-)}$ *mice exposed to seminal fluid from wildtype mice containing Neu5Gc-rich CD52,* are both targeted by antibodies and are increasingly phagocytosed by female uterine immune cells (75). These insights have potential relevance for human fertility where Neu5Gc or anti-Neu5Gc antibodies in the reproductive tract are common among infertility patients, but not healthy controls (29).

In addition to blocking fertilization, it is possible that anti-Neu5Gc immunity from a primed $CMAH^{(-/-)}$mother (29) could also negatively affect a $CMAH^{(+/-)}$embryo or fetus in a manner similar to hemolytic diseases of the newborn caused by ABO glycan mismatches.

Reproductive xenosialitis could thus be a plausible mechanism mediating directional selection, leading to the fixation of the loss-of-function allele in the population, irrespective of the mechanism(s) involved for the initial selection favoring the mutation (see **Figure 4**).

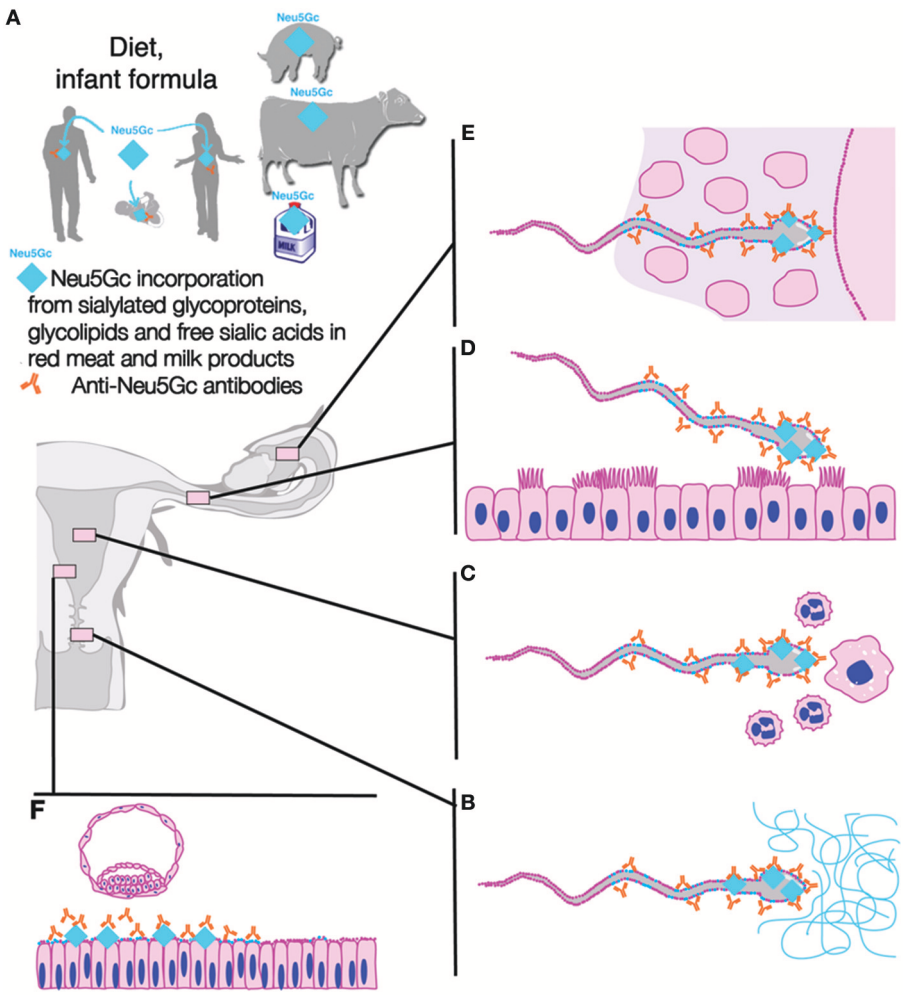

FIGURE 4 | Exogenous (dietary) Neu5Gc and anti-Neu5Gc antibody as contributing factors to unexplained human infertility. The combination of incorporated dietary xenoglycan Neu5Gc (even in trace amounts) from red meat and milk products including cow milk-based infant formula and anti-Neu5Gc antibodies in males or females **(A)** could have deleterious consequences on human fertility via a number of potentially additive mechanisms which include: **(B)** coating of sperm by male and/or female anti-Neu5Gc antibodies, interference with sperm function, including passage through cervical mucins and/or other restrictive parts of the female reproductive tract such as the utero-tubal junction, **(C)** increased sperm death via female cellular and humoral immunity, where most sperm are killed by female immunity, **(D)** interference with sperm capacitation when sperm membranes get dynamically reconfigured, **(E)** interference with sperm penetration of vestment, interference with sperm-egg interactions, **(F)** interference with endometrial decidualization and receptivity resulting in reduced success of implantation. Figure modified from Ma et al. (75) with permission.

CONCLUSIONS AND PERSPECTIVES

It is interesting that watershed events, such as the loss of Neu5Gc from the glycocalyx of human cells have occurred numerous times in many mammalian and other vertebrate species. These cases of convergent evolution represent precious opportunities for increased understanding of evolutionary processes. In some respects, Neu5Gc is an ideal self-molecule as it is "private" to vertebrates and, based on current data, has yet to be successfully mimicked by microbes. Against the background of this benefit, the loss of Neu5Gc appears paradoxical and may implicate strong selective regimes, either catastrophically caused by pathogens, or under directional sexual selection via female immunity to paternal xenoglycans. Massive genetic drift, or combinations of milder selection and founder events, can also not be excluded.

More information on species expected to encounter Neu5Gc in their diets, i.e., mustelids, pinnipeds, and humans, is needed to begin answering several outstanding questions in the field: For instance, what are the potential protective functions of anti-Neu5Gc antibodies in species that lack this sialic acid, especially as regards ongoing protection from cross-species infections by enveloped viruses bearing Neu5Gc on their viral envelopes? Or on the flip-side, what are the potential liabilities of anti-Neu5Gc antibodies due to autoimmunity against incorporated dietary Neu5Gc? Evolutionary events such as the ones discussed here exemplify how glycans, rather than representing the end result of different evolutionary histories and contingencies, can become an evolutionary force of their own and constrain future evolution of entire lineages including subsequent compensatory evolution of glycan binding immune receptors (15, 76).

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MA and PG did the research and wrote the paper. PG produced the figures.

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Exposure of Children to Rural Lifestyle Factors Associated With Protection Against Allergies Induces an Anti-Neu5Gc Antibody Response

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Rural lifestyle has been shown to be highly protective against the development of allergies. Contact to farm-animals or pets and early-life consumption of milk products turned out to be important. These exposures provide contact to N-glycolylneuraminic acid (Neu5Gc), a sialic acid naturally expressed in mammalians but not in humans or microbes although both are able to incorporate exogenously provided Neu5Gc and induce thereby an anti-Neu5Gc antibody response. Farmers' children had elevated levels of anti-Neu5Gc antibodies associated with increased contact to Neu5Gc. Farm-related exposures that were associated with protection against allergies such as exposure to farm-animals or pets and consumption of milk were also associated with an antibody response to Neu5Gc in children. Exposure to cats was associated with increased anit-Neu5Gc IgG levels at different timepoints assessed between 1 year of age and school-age. Moreover, consumption of non-pasteurized milk in the first year of life was associated with increased anti-Neu5Gc IgG levels. Neu5Gc-providing exposures that were associated with protection against allergies were reflected in an elevated anti-Neu5Gc IgG level in children. Exposure to Neu5Gc was associated with anti-inflammation and protection of asthma development in children and mice without contribution of anti-Neu5Gc antibodies.

Keywords: N-glycolylneuraminic acid, asthma, regulatory T cells, anti-inflammatory, animal contact, hygiene hypothesis

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The term "hygiene hypothesis" has emerged to describe the correlation between the increase in hygienic conditions and the elevation in allergic disorders and asthma, but also in autoimmune and inflammatory diseases in westernized countries over the last decades (1). The hygiene hypothesis was proposed after a lower risk of hay fever and atopic sensitization among children having more siblings was observed (2). The protective effect was assigned to an altered exposure to microbes during childhood. Other findings supported this hypothesis: early entry to day care of the children had a protective effect against the development of allergies and Italian military students with antibodies to hepatitis A virus had a lower prevalence of atopy and atopic respiratory diseases (3, 4). More recent studies have consistently linked altered exposures to microbes early in life with later life risk of allergy and asthma (5). Further, rural lifestyle, especially farm environment,

turned out to be highly protective (6–11). We found that, in addition to microbial exposure, rural lifestyle also provide increased exposure to non–microbial-derived N-glycolylneuraminic acid (Neu5Gc) which was reflected by increased levels of anti-Neu5Gc antibodies (12). Although detailed underpinning immunological mechanisms were not fully known, this was the first study showing an immune-modulatory, anti-inflammatory effect of Neu5Gc itself, which is in contrast to findings of xenograft rejection and tumor growth where anti-Neu5Gc antibodies were associated with a pro-inflammatory status (13–19).

Neu5Gc is a specific marker for non-human cells and proteins. Neu5Gc is a sialic acid composed of nine carbon sugars found at the most outer unit of the cellular glycocalyx and on secreted glycoproteins in most mammalian cells. In contrast to all other mammals, including primates, humans and new world monkeys lack the enzyme CMP-Neu5Ac hydroxylase (CMAH) due to a mutation in the respective gene and are therefore not able to synthesize Neu5Gc from the precursor N-acetylneuraminic acid (Neu5Ac) (20–22). Humans are able to uptake Neu5Gc via fluid pinocytosis and a specific lysosomal transporter and incorporate it in newly synthesized glycoproteins. Known dietary sources of Neu5Gc are red meat and cow's milk products (23–25). As a consequence, humans mount a humoral immune response upon exposure to other mammalian cells by producing anti-Neu5Gc immunoglobulins (26, 27).

Rural lifestyle factors described to be protective against the development of allergies and asthma are contact to more and more-diverse microbes in the environment, contact to farm-animals or pets, and altered nutrition such as consumption of more milk products (9, 11, 28–31). To investigate which of the protective farm-exposures is associated with induction anti-Neu5Gc antibodies, we measured anti-Neu5Gc IgG in sera of children from a random sample of the cross-sectional PARSIFAL study (Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle) ($n = 299$) including school-age children and from the longitudinal PASTURE study (Protection against Allergy-Study in Rural Environments) including serum samples from cord blood ($n = 836$), 1 year ($n = 734$), 4.5 years ($n = 700$), and 6 years ($n = 728$). Anti-Neu5Gc IgG levels were assessed by quantifying anti-Neu5Gc-polyacrylamid IgG corrected by subtraction of anti-Neu5Ac-polyaccrylamid IgG, which is certainly an underestimation of the anti-Neu5Gc response (23, 26, 32). Anti-Neu5Gc IgG levels were associated with rural environmental exposures reflecting protection against allergies and asthma (contact to farm-animals assessed by the presence in facilities related to livestock, such as stables and contact to pets; nutritional factor such as consumption of cow's milk; contact to microbes assessed by levels of endotoxin and extracellular polysaccharide in children's mattresses) (**Table 1**).

Contact to animals specially contact to cats was strongly and consistently associated with increased anti-Neu5Gc IgG antibodies. Contact to cats was associated with elevated antibody levels at different timepoints assessed between 1 year of age and school-age, while contact to a stable environment was significantly associated with anti-Neu5Gc IgG levels in the school-age children of the cross-sectional study and in 4.5 years old children of the longitudinal study (**Table 1**). Worth mentioning is that children having contact to cats had a reduced risk to develop asthma later in life [adjusted odds ratio (aOR) for exposure to cat during pregnancy and asthma at 6 years: 0.6 (95% confidence interval (CI) 0.36–1); aOR for exposure to cat in the first year of life and asthma at 6 years: 0.59 (95% CI 0.35–0.98); aOR for exposure to cat at 4 years of age and asthma at 6 years: 0.52 (95%CI 0.31–0.87)]. Moreover, cow's milk consumption was significantly associated with increased anti-Neu5Gc IgG levels at 1 year of age. Remarkable, consumption of non-pasteurized milk was much stronger associate with anti-Neu5Gc antibody titers compared to pasteurized milk consumption, although we don't know if pasteurization reduces the immunogenicity of Neu5Gc bearing proteins (**Table 1**). Finally, exposure to microbial components such as endotoxin or extracellular polysaccharide in mattresses of the school-age children was not significantly associated with anti-Neu5Gc IgG levels.

The data outlined above show that exposure to rural lifestyle factors, which provide protection against the development of allergies or asthma and additionally provide contact to Neu5Gc such as contact to cats or early-life consumption of farm-milk induced an antibody response against Neu5Gc. Contact to microbes, not expressing Neu5Gc was not associated with anti-Neu5Gc levels. Elevated anti-Neu5Gc IgG levels were associated with dietary Neu5Gc intake and infections with certain viruses such as Epstein-Barr virus (13, 33). Our data indicate that also inhaled Neu5Gc provided by contact to cats induced an anti-Neu5Gc antibody response, although it is still possible that inhaled antigens reach the intestine.

The route of initial antigen exposure is crucial in determining whether tolerance or allergic sensitization occurs (34). In contrast to early-life exposure of antigens via the skin, which has been proposed as a key route for allergic sensitization to allergens, exposure via the oral route is associated with induction of tolerance. Inhaled antigens reaching the intestine or orally ingested antigens are encountered by the intestinal-draining lymph nodes which is also the site of antigen-specific regulatory T cell generation a crucial event for tolerance induction (34). Induction of tolerance is one key mechanism how environmental factors might protect children from the development of allergies and asthma (28, 30, 35). We have recently shown that Neu5Gc-feeding of mice had protective effects in models of airway and gut inflammation in the absence of anti-Neu5Gc antibodies (12). The beneficial effect of Neu5Gc was based on anti-inflammatory effects such as increased numbers of regulatory T cells and elevated expression of indoleamine 2,3-dioxygenase (IDO), retinaldehyde dehydrogenase 2 (RALDH2), and IL-10 in dendritic cells and on a reduction of T helper cell type 17 responses (12). Moreover, Massoud et al. showed that intravenous immunoglobulin therapy was able to attenuate airway hyperresponsiveness and inflammation mediated by regulatory T cells in a model of airway inflammation in mice. This effect was dependent on intravenous immunoglobulin sialylation, because neuraminidase-treated intravenous immunoglobulin was not able to induce regulatory T cells and intravenous

TABLE 1 | Association between farm-related exposures and anti-Neu5Gc IgG levels.

	Longitudinal§				Cross-sectional°
GMR [95% CI]	**Cord blood**	**1 year**	**4.5 years**	**6 years**	**School-age**
Contact to animal-related exposure					
Stable contact [1]	0.77 [0.25, 2.36]	0.78 [0.15, 4.02]	**5.54 [1.34, 22.92]***	1.26 [0.39, 4.14]	**3.79[1.7, 8.45]*****
Contact to pets:[1]					
Cats	1.59 [0.65, 3.91]	**14.61 [2.06, 103.68]****	**3.72 [1.34, 10.37]***	**5.50 [2.13, 14.22]*****	**1.71 [1.14, 2.57]****
Dogs	2.23 [0.93, 5.37]	2.37 [0.24, 23.84]	1.13 [0.40, 3.19]	0.70 [026, 1.85]	1.57 [0.88, 2.8]
Nutrition					
Non-pasteurized vs. pasteurized milk consumption [1]	0.88 [0.32, 2.40]	**23.92 [5.13, 111.57]*****	1.60 [0.52, 4.97]	1.49 [0.52, 4.3]	1.43 [0.9, 2.27]
Cow's milk vs. no-milk consumption [1]		**5.57 [1.67, 18.58]****	0.50 [0.15, 1.73]	0.63 [0.17, 2.34]	
Microbal exposure					
Endotoxin					1.12 [0.95, 1.31]
Extracellular polysaccharide					1.11 [0.97, 1.27]

§GMR (geometric mean ratio), adjusted for center, farmer, parental atopy, sex, and duration of breastfeeding.
[1]Cord blood: associations with exposure during pregnancy; 1 year, 4.5 years, and 6 years: associations with exposure during the last 12 months; for exposure during the first year of life, adjustment for prenatal exposure.
°GMR. Variables: adjusted for farming, sex, and age.
Bold values were statistically significant.
Two-sided P < 0.05 were considered significant.
**P < 0.05; **P < 0.01; ***P < 0.001.*

immunoglobulin enriched in sialic acid displayed an enhanced anti-inflammatory activity. Furthermore, the authors showed that the anti-inflammatory effect of sialylated intravenous immunoglobulin was mediated by binding to the C-type lectin dendritic cell immunoreceptor (DCIR) (36, 37).

Other mechanisms underpinning the protective effect of Neu5Gc contact might be mediated by Neu5Gc binding to Sialic acid-binding Ig-like lectins (Siglecs). Siglecs have an intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM) thereby inhibiting activation signals by activation of Src homology region 2 domain-containing phosphatase (SHP)-1 and−2, tyrosine phosphatase, and suppressor of cytokine signaling (SOCS)-3 (38–40). Especially, activation of Siglec-8 on eosinophils by sialic acids turned out to be a promising target for asthma treatment because of induction of apoptosis of those cells (41). These data indicate that exposure to Neu5Gc might support tolerance development.

We suggest that Neu5Gc behaves as an anti-inflammatory molecule in the human immune system that is able to prevent the development of asthma symptoms but also colitis. The loss of Neu5Gc during human evolution, possibly driven by selective pressure of a pathogen, may have removed this anti-inflammatory molecule and consequently removed one of the "brakes" previously used to limit immune pathology (42). Exposure to animals or animal-derived foods containing Neu5Gc seems to replace this molecule and assist immune regulatory processes. However, the move away from traditional farming environments limits the exposure to Neu5Gc and thereby might contribute to exaggerated inflammatory responses. Future studies should consider the deliberate exposure to Neu5Gc as a novel anti-inflammatory strategy but also involve studies using Neu5Gc together with anti-Neu5Gc antibodies since there are studies showing a pro-inflammatory role of Neu5Gc in combination with anti-Neu5Gc antibodies in tumor and xenograft models (13–19).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of guidelines of the ethics committee of the canton St. Gallen with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ethics committee of the canton St. Gallen.

AUTHOR CONTRIBUTIONS

RFr, CR, RFe, LO, and RL conceived and designed the experiments, discussed the data, and wrote the paper.

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REVIEW
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The Structural Complexity and Animal Tissue Distribution of *N*-Glycolylneuraminic Acid (Neu5Gc)-Terminated Glycans. Implications for Their Immunogenicity in Clinical Xenografting

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N-Glycolylneuraminic acid (Neu5Gc)-terminated glycans are present in all animal cells/tissues that are already used in the clinic such as bioprosthetic heart valves (BHV) as well as in those that potentially will be xenografted in the future to overcome end stage cell/organ failure. Humans, as a species lack this antigen determinant and can react with an immune response after exposure to Neu5Gc present in these products/cells/tissues. Genetically engineered source animals lacking Neu5Gc has been generated and so has animals that in addition lack the major αGal xenoantigen. The use of cells/tissues/organs from such animals may improve the long-term performance of BHV and allow future xenografting. This review summarizes the present knowledge regarding structural complexity and tissue distribution of Neu5Gc on glycans of cells/tissue/organs already used in the clinic or intended for treatment of end stage organ failure by xenografting. In addition, we briefly discuss the role of anti-Neu5Gc antibodies in the xenorejection process and how knowledge about Neu5Gc structural complexity can be used to design novel diagnostics for anti-Neu5Gc antibody detection.

Keywords: *N*-glycolylneuraminic acid, xenograft, bioprosthetic heart valve, carbohydrate antigen, anti-carbohydrate antibodies, carbohydrate epitope

INTRODUCTION

Products isolated from animal tissues have been used in clinical medicine for a long time as exemplified by porcine insulin introduced in the 1920's and bioprosthetic heart valves (BHV) in 1965 (Binet et al., 1965). In recent years, focus has also been on the potential use in humans of live cells and tissues from animals, primarily pigs, to overcome the shortage of human cells/organs for transplantation (Auchincloss and Sachs, 1998; Cowan and Tector, 2017; Ekser et al., 2017). A major obstacle for transplantation of live animal tissue into humans is the strong xenogeneic immune rejection initiated in the recipient (Auchincloss and Sachs, 1998; Cowan and Tector, 2017; Ekser et al., 2017). The most immediate barrier preventing grafting of porcine tissues into man

and non-human primates was shown to be explained by preformed antibodies specific for the Galα1,3Gal (αGal) carbohydrate determinant present on cell surface glycoconjugates (Auchincloss and Sachs, 1998; Ezzelarab et al., 2005). These αGal specific antibodies cause hyperacute rejection of vascularized porcine tissues in humans and non-human primates similar to that caused by preformed anti-blood group ABO antibodies in human allotransplantation (Holgersson et al., 2005). In addition, several non-αGal antigens that humans can develop antibodies against including *N*-glycolylneuraminic acid (Neu5Gc), have been identified and they may contribute to the xeno-rejection process (Ezzelarab et al., 2005; Byrne et al., 2011; Padler-Karavani and Varki, 2011; Galili, 2012; Miyagawa et al., 2012; Salama et al., 2015).

This review summarizes the present knowledge regarding the structural complexity and distribution of Neu5Gc on glycans of BHV as well as cells/organs intended for treatment of end stage organ failure by xenografting. In addition, we discuss how we can use our knowledge regarding Neu5Gc structural complexity for the design of novel diagnostics for anti-Neu5Gc antibody detection. The possible significance of anti-Neu5Gc antibodies in the xenorejection process has been the subject of recent reviews (Padler-Karavani and Varki, 2011; Salama et al., 2015) and will only be commented on briefly in this contribution.

CHEMICAL STRUCTURE DIVERSITY OF SIALIC ACIDS FOCUSED ON Neu5Gc

Sialic acids are α-keto acids with a nine-carbon backbone and are normally placed terminally in the reducing end of glycans (Angata and Varki, 2002; Schauer, 2004). They are found in the deuterostome lineage, i.e., chordates and echinoderms (e.g., sea stars), of animals and in certain bacteria (Angata and Varki, 2002; Schauer, 2004). Sialic acid used to be considered a synonym for *N*-acetylneuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid; Neu5Ac), but since its discovery in the 80's the deaminated neuraminic acid, KDN (2-keto-3-deoxy-*D*-glycero-*D*-galacto-nononic acid), is also included in the family of sialic acids (Inoue and Kitajima, 2006). Like *N*-acetylneuraminic acid, KDN is also found in vertebrates and bacteria. The structural diversity among sialic acids is vast with more than 50 distinct molecules that are biosynthetic derivatives of either *N*-acetylneuraminic acid or KDN (Angata and Varki, 2002; Schauer, 2004). *N*-glycolylneuraminic acid (Neu5Gc) is another major type of sialic acid and is also expressed in deuterostomes. The initial characterization of Neu5Gc biosynthesis was explored by Schauer in the 1960's showing that Neu5Ac was converted by CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) to the *N*-glycolyl form by addition of an oxygen atom to the *N*-acetyl group (Schauer et al., 1968; Schauer, 1991) illustrated in **Figure 1**. Birds, reptiles, amphibians, sperm whales, and several other species including New World monkeys and humans lack CMP-*N*-acetylneuraminic acid hydroxylase and therefore these species lack Neu5Gc (Peri et al., 2017). However, trace amounts of Neu5Gc have been identified in humans, a finding explained by an uptake from ingested meat and dairy products (Schauer et al., 1968; Tangvoranuntakul et al., 2003).

GENERAL ASPECTS OF GLYCOCONJUGATES AND ANTI-CARBOHYDRATE ANTIBODIES

The Structural Diversity of Cell Surface Glycoconjugates

The surface of every cell is covered with a diverse array of glycans, carried by proteins or lipids in the outer plasma membrane leaflet, mediating interactions leading to cell adhesion, trafficking, and signaling (Gustafsson and Holgersson, 2006; Sperandio et al., 2009). Glycans determine self/non-self as they are targets for antibodies of clinical significance in transfusion medicine and transplantation (Holgersson et al., 2005; Gustafsson and Holgersson, 2006). Furthermore, cell surface carbohydrates constitute important attachment sites for viruses, bacteria and bacterial toxins and as such they are required by microbes to initiate infection (Karlsson, 2001; Gustafsson and Holgersson, 2006).

Glycosylation is a common post-translational modification (PTM) of proteins involving enzymatic glycosylation of the protein backbone (Kobata, 2004). The varying sequence and chain length as well as the anomeric configuration (α or β), linkage position and branching sites make glycosylation the structurally most diverse PTM (Dwek, 1995). Covalent modifications of individual sugar residues by sulfation, phosphorylation, acetylation, or methylation add further structural variation to the carbohydrate chain. Therefore, the structural diversity that can be obtained in glycan chains is by far exceeding the complexity obtained by amino acids in polypeptides (Samuelsson and Breimer, 1987).

Two of the most abundant protein glycosylation forms are *N*- and *O*-linked glycosylation. *N*-linked glycans are usually attached via an *N*-acetylglucosamine (GlcNAc) to Asparagine (Asn). They are classified into three types, the high mannose (oligomannose), complex, and hybrid types. *N*-glycan biosynthesis is initiated via the synthesis of the $Man_5GlcNAc_2$ core unit on the dolichol pyrophosphate lipid anchor, which is then re-oriented to the luminal side of the endoplasmic reticulum (ER) membrane and extended to a $Glc_3Man_9GlcNAc_2$ sequence. Transfer of the $Glc_3Man_9GlcNAc_2$ oligosaccharide to the consensus sequence (N-X-S/T) in acceptor polypeptides is performed en bloc by the oligosaccharyltransferase (OST). *N*-glycans are further modified in the late ER and Golgi apparatus generating a plethora of *N*-glycan structures. The processing is possibly determined by the function of the glycan structures and the compartment where they are localized, resulting in a species- or even cell type-specific diversity of *N*-linked glycans (Schwarz and Aebi, 2011; Aebi, 2013).

Mucin-type *O*-linked glycans are attached to Ser or Thr via *N*-acetylgalactosamine (GalNAc), but other *O*-glycans may be linked to Ser/Thr via GlcNAc, fucose, glucose, mannose, or xylose (van den Steen et al., 1998). *O*-glycan biosynthesis is initiated in the ER and the chain is further extended in the ER and

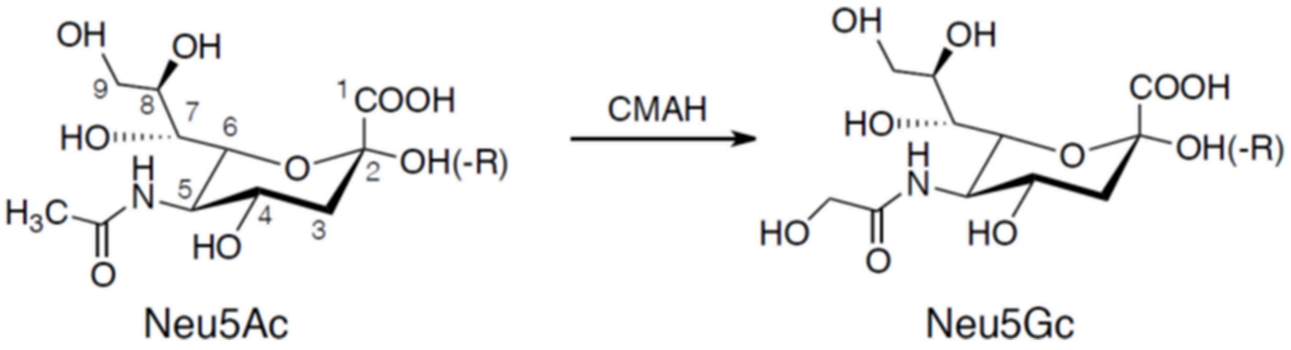

FIGURE 1 | Chemical structures of Neu5Ac and Neu5Gc. Neu5Gc is generated from Neu5Ac by the enzyme CMP-*N*-acetylneuraminic acid hydroxylase (CMAH). Neuraminic acids are linked to the carbohydrate core chain (-R) by a glycosidic linkage involving the hydroxyl group at carbon atom 2 forming an α2-3 or α2-6 linkage. A second neuraminic acid can be added to the penultimate neuraminic acid by an α2-8 linkage.

Golgi by a stepwise addition of monosaccharides. There is no known consensus sequence for initiation of *O*-glycosylation. The initiating step of mucin-type glycosylation is the addition of the GalNAc monosaccharide from UDP-GalNAc to the hydroxyl groups in serine and threonine residues; a reaction catalyzed by a large family of up to 20 different polypeptide GalNAc-transferases (ppGalNAc-Ts) (Bennett et al., 2012). Three distinct regions are recognized in *O*-linked glycans and include the two or three innermost sugar residues nearest the peptide chain constituting the core region, the backbone region contributing to *O*-glycan chain length, and the terminal region with its bioactive determinants (Hanisch, 2001). The determinants are often sialylated, sulfated, acetylated, and/or fucosylated. At least eight different *O*-glycan core chain types, of which cores 1–4 are more common than the rare cores 5–8, have been identified in mammalian glycoproteins. All are based on the innermost αGalNAc residue, which is further substituted at the C3, C6, or both positions (Hanisch, 2001).

Glycolipids are mainly found in the plasma membrane with the lipophilic part (ceramide) integrated in the outer layer of the lipid bilayer and the saccharide chain exposed to the cell environment. In contrast to glycoproteins that carry several different saccharide chains, only one single glycan is attached to each ceramide. As for protein-linked glycoconjugates, glycolipid structural complexity is vast. Immunogenic determinants are linked to various core saccharide chain types (ganglio-, globo-, lacto-, neolacto-series etcetera) (Holgersson et al., 1992). Sialic acid-containing glycolipids (gangliosides) are based on different saccharides of which lactosylceramide and ganglio-series compounds are most abundant.

Structural Diversity of Neu5Gc-Terminated Glycans

Sialic acids including Neu5Gc are mostly found terminally on glycan chains of glycoproteins and glycolipids. They are commonly linked via an α2,3- or α2,6-linkage to Gal, an α2,6-linkage to GalNAc, or via an α2,8-linkage to another sialic acid (Angata and Varki, 2002; Schauer, 2004). Glycans with the sialic acid linked to other sugar residues and in other binding positions exist (Angata and Varki, 2002; Schauer, 2004). For details regarding the chemical structure of various neuraminic acid-containing glycans, the reader is referred to previously published reviews and text books (Angata and Varki, 2002; Schauer, 2004; Varki et al., 2017).

A variety of Neu5Gc-terminated *N*- and *O*-glycans have been identified. Using CHO-K1 cells as host cells and a mucin-type fusion protein as a reporter protein to study *O*-glycosylation, sialylated core 1, core 2, core 3, and extended core 1 *O*-glycans were identified following transient co-expression of the different core enzymes in CHO-K1 cells (Liu et al., 2015). Between 5 and 10% of the sialylated *O*-glycans carried Neu5Gc and it was found α2,3- and α2,6-linked (following expression of ST6Gal I) to Gal and α2,6-linked to GalNAc (Liu et al., 2015). Choi and co-workers used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to study *N*-glycans released from native porcine heart valves or heart valves treated with α-galactosidase (Choi et al., 2012). They identified a number of complex type *N*-glycans carrying Neu5Gc (Choi et al., 2012). The full extent of the structural diversity of *N*- and *O*-glycans carrying Neu5Gc remains to be elucidated. However, a not too brave assumption is that the majority of glycans carrying Neu5Ac have their Neu5Gc counterpart.

The most common Neu5Gc-terminated glycolipid is the GM3 ganglioside with Neu5Gc linked to lactosylceramide (Iwamori and Nagai, 1978; Gasa and Makita, 1980; Hanagata et al., 1990). Complex Neu5Gc-containing gangliosides with several sialic acids have been identified (Ohashi and Yamakawa, 1977; Ariga et al., 1983; Nakao et al., 1991), also in various combinations with blood group ABO and Lewis antigen determinants (van Dessel et al., 1979; Nohara et al., 1990). Terminal sialic acid disaccharides exist in all the possible combinations NeuGc-NeuGc-, NeuAc-NeuAc-, NeuGc-NeuAc-, and NeuAc-NeuGc- (Watarai et al., 1991).

Recognition of Saccharide Structures by Anti-carbohydrate Antibodies

Traditionally, carbohydrates have been considered T lymphocyte-independent antigens because they activate B

lymphocytes without T-cell help. As most carbohydrates cannot be presented via MHC class II antigens and, thus, not recruit T-cell help, the B-cell response lack affinity maturation and is skewed toward the production of IgM and IgG2 antibodies in human (Vos et al., 2000). To overcome the lack of T-cell help in the response of B-cells to carbohydrate antigens, neoglycoconjugates have been developed by coupling the carbohydrate antigen to carrier proteins. Upon intracellular processing, peptides from the latter can be presented by MHC class II antigens on B-cells to T-cells that upon activation can provide help to the B-cell. A good example of this is the *Haemophilus influenzae* neoglycoconjugate vaccine (Micoli et al., 2018). Polysaccharides carrying both negatively and positively charged substituents have been shown to interact with MHC class II species (Avci and Kasper, 2010), as have oxidative breakdown products of polysaccharides (Velez et al., 2009). Anti-carbohydrate antibodies are normally of low affinity, often of 10^3-10^5 times less affinity than anti-peptide or -protein antibodies (Krause and Coligan, 1979; MacKenzie et al., 1996; Brorson et al., 2002). The low affinity is compensated for by a high avidity provided for by the decavalent configuration of the IgM antibody or self-associated IgG2 antibodies in humans (Greenspan et al., 1988; Cooper et al., 1991). Multivalently configured, as in IgM or self-assembled IgG2, anti-carbohydrate antibodies facilitate high avidity binding to multivalently expressed or clustered carbohydrate antigens on the surface of cells, bacteria, and viruses. They are thus ideally suited to distinguish cells expressing high densities of a carbohydrate antigen from those expressing low densities of the same antigen.

The low affinity of anti-carbohydrate antibodies (and lectins) as opposed to anti-peptide antibodies may be explained by the contribution of entropic factors to binding, which is not solely reliant on enthalpic factors (reviewed in Haji-Ghassemi et al., 2015). Because of the flexible nature of carbohydrates, antibody binding requires unfavorable immobilization of otherwise flexible parts of the saccharide chain and, thus, loss of entropy (Haji-Ghassemi et al., 2015). Therefore, extension of the sugar chain and fixation of the anomeric carbon in one conformation may increase antibody binding affinity even if the extending sugar is not involved in the binding (Haji-Ghassemi et al., 2015). Further, the entropic consequences of water in binding of anti-carbohydrate antibodies are hard to predict because solvating water molecules may need to be displaced or trapped during antibody-antigen complex formation (Haji-Ghassemi et al., 2015).

Early studies on the structural features of anti-carbohydrate antibodies suggested that the antibody binding site could encompass up to six monosaccharide residues and to be pocket- or groove-shaped (Kabat, 1978). Pocket-shaped for binding determinants placed terminally in the saccharide chain and groove-shaped for binding internally on polysaccharide structures. In their comprehensive review, Haji-Ghassemi and co-authors concluded after reviewing the structural features of anti-carbohydrate antibodies specific for over 20 antigens, that even though they share characteristic features there are no general rules governing their behavior (Haji-Ghassemi et al., 2015).

The crystal structure of the Fab fragment of the murine anti-Neu5Gc antibody has been resolved at a 2.2 Å resolution and a molecular model of this fragment in complex with the saccharide moiety of *N*-glycolyl GM3 ganglioside has been generated (Krengel et al., 2004; Bjerregaard-Andersen et al., 2018).

STRUCTURAL COMPLEXITY AND SPECIES/TISSUE DISTRIBUTION OF Neu5Gc IN TISSUES OF RELEVANCE FOR BIOPROSTHETIC HEART VALVES

Several types of bio-devices of animal origin have been developed for clinical use. Examples of these are sheets to build up the abdominal wall in the repair of hernias (Patel et al., 2018) and BHV to replace diseased heart valves (Fiedler and Tolis, 2018). BHV used clinically are mainly produced from bovine, porcine, and equine tissues such as pericardium and heart valves. The tissues are processed, encompassing for example glutaraldehyde, ethanol, and anti-calcification, to reduce immunogenicity and to extend preservation times of the tissues. Carbohydrates are resistant to many of these treatments as shown by remaining αGal antigens in commercial BHV products (Kasimir et al., 2005; Naso et al., 2013). Sialic acids are negatively charged ("acidic" carbohydrate components) and are slightly more sensitive to chemical degradation compared to neutral saccharide components. However, sialic acid-terminated saccharides have been identified by immunohistochemistry in formaldehyde-fixed tissue sections (Morozumi et al., 1999; Magnusson et al., 2005) and a recent study did not find any change in anti-Neu5Gc staining of naïve and glutaraldehyde-treated (0.02–2%) porcine valves indicating that these saccharides may resist the processing treatments (Lee et al., 2016). However, BHVs available for clinical use contain extremely small amounts of biological tissue and are very expensive, why it is difficult to perform structural investigations on antigen expression using chemical methods. Therefore, studies on native animal pericardium and heart valve tissues have been performed to make a chemical characterization possible. Bearing in mind that carbohydrate determinants, at least in part, remain intact despite the processing of the tissue.

Valve Cusps

Immunohistochemical analysis of naïve porcine aortic valve cusps showed a strong Neu5Gc staining of the cusp endothelium (Reuven et al., 2016). Using immunohistochemistry, Lee and coworkers tested pig heart valves obtained from wild-type, GTKO/CD46 and GTKO/CD46/NeuGcKO animals and a strong Neu5Gc expression was found in wild-type and GTKO/CD46 tissues that was absent in the GTKO/CD46/NeuGcKO valves (Lee et al., 2016).

Terminal Neu5Gc saccharides (assumed to be the Hanganutziu-Deicher, HD, antigens) have been identified by mass spectrometry in *O*-glycans isolated from naïve pig aortic and pulmonary valves (Jeong et al., 2013). A more complex pattern of Neu5Gc-terminated saccharides was found in the aortic valves compared to the pulmonary valves and the heart muscle.

In investigations of glycolipids of naïve animal heart valves and pericardia, an unexpected finding was the lack of Neu5Gc-terminated gangliosides in pig heart valves (Barone et al., 2014), while the pig, bovine, and equine pericardia all contained gangliosides with terminal Neu5Gc residues (Barone et al., 2018). Neu5Gc-GM3 was found in all animal species while other gangliosides showed a species-specific distribution; Neu5Gc-GD3 (equine), Neu5Gc-GM1 (pig, bovine), Fuc-Neu5Gc-GM1 (pig). These structures were deduced by a combination of thin-layer chromatographic mobility, staining by the HD antigen-specific chicken monoclonal antibody (HU/Ch2-7; Asaoka et al., 1992) in combination with liquid chromatography-mass spectrometry of purified ganglioside fractions (Barone et al., 2018).

Pericardium

Immunohistochemical analysis of naïve porcine and bovine pericardia showed anti-Neu5Gc staining of the matrix of the pericardium as well as the endothelium of a small artery and a capillary (Reuven et al., 2016). A strong Neu5Gc expression was found in wild-type and GTKO/CD46 pig pericardium while pericardia from GTKO/CD46/NeuGcKO animals were negative (Lee et al., 2016).

Studies on BHV Used in the Clinic

The commercial BHVs used in the clinic are mainly produced from bovine pericardia even if some manufacturers use porcine valves and porcine as well as equine pericardia (Reuven et al., 2016). Immunostaining and HPLC analysis of homogenates from six different commercial BHV revealed presence of Neu5Gc in all products but the limited amount of tissue did not allow any further exploration of saccharide structures (Reuven et al., 2016). In another study, three different commercial BHV valves were tested and all showed strong anti-Neu5Gc binding as well as binding of human serum (Lee et al., 2016).

STRUCTURAL COMPLEXITY AND SPECIES/TISSUE DISTRIBUTION OF Neu5Gc IN TISSUES OF RELEVANCE FOR XENOTRANSPLANTATION

Endothelial Cells

Flow cytometric analysis using the HU/Ch2-7 antibody specific for HD antigens revealed strong expression of HD antigens in cultures of porcine and bovine aortic endothelial cells and immunohistochemical analysis of porcine kidney revealed strong expression in all vascular endothelial cells (Morozumi et al., 1999; Reuven et al., 2016). Also, pericardial vessel endothelium contained Neu5Gc glycans (Reuven et al., 2016).

Bouhours and co-authors studied gangliosides from primary cultures of porcine endothelial cells labeled with ^{14}C-monosaccharides and were able to identify the GM3 and GD3 compounds with *N*-glycolylneuraminic acid as their predominant sialic acid (Bouhours et al., 1996).

Even if not all animal organs corresponding to the vascularized organs currently used in clinical transplantation have been analyzed for Neu5Gc expression in the specific organ, it can be anticipated that endothelial cells of these organs express Neu5Gc as shown for pig kidney endothelium (Reuven et al., 2016).

Pancreatic Islets

Glycoproteins carrying *N*-linked HD determinants have been identified in adult pig islet cells together with several other sialic acid-capped compounds that reacted with human natural antibodies (Komoda et al., 2004). In addition, porcine pancreas was shown to contain gangliosides with Neu5Gc (Nakamura et al., 1984).

Cornea

Our knowledge regarding corneal xenotransplantation has increased considerably and corneal grafting is, together with pancreatic islets, close to be tested in human clinical trials. Neu5Gc have been identified by immunohistochemistry in all layers of pig cornea (Cohen et al., 2014). Mass spectrometric analyses of pig corneal endothelial cells and keratocytes releveled several *N*-glycans with terminal Neu5Gc (Kim et al., 2009). Because cornea is a non-vascularized tissue, the clinical relevance of Neu5Gc antigen expression in this tissue remains to be elucidated.

Lymphocytes

During reperfusion of grafted vascularized organs, considerable amounts of blood cells, including leukocytes, trapped in the organ are transferred to the recipient and may induce an immune response. Leukocytes remain in the harvested organs despite extensive rinsing of the vascular tree with perfusion solution (Magnusson et al., 2003). Therefore, knowledge regarding carbohydrate antigen expression also in lymphocytes is of importance.

Porcine spleen lymphocytes contain a complex ganglioside mixture with Neu5Gc-GM3 and -GD3 as major constituents (Hueso et al., 1985), while the ganglioside mixture of peripheral blood lymphocytes was less complex with Neu5Gc-GD3 as the major ganglioside species (Hueso et al., 1985; Magnusson et al., 2003).

Studies on peripheral blood lymphocytes and thymocytes of calves revealed GM3 as major component and that 97% of the gangliosides from peripheral cells contained Neu5Gc, while the ganglioside composition of thymic cells was more complex containing several ganglioside species including Neu5Ac sialic acids (Dyatlovitskaya et al., 1980).

Vascularized Organs

Most studies identifying Neu5Gc antigens in animals have been performed on mouse, bovine, rabbit, and sheep tissues. Studies on vascularized organs of pigs, the most likely species to be used for xenografting, are limited. However, Neu5Gc-containing gangliosides have been structurally characterized in porcine plasma (Hanagata et al., 1990), skeletal muscle (Ariga et al., 1983), adipose tissue (Ohashi and Yamakawa, 1977), peripheral nerve (Magnusson et al., 2005), small intestine (Diswall et al., 2007, 2014), kidney (Diswall et al., 2007), and

pancreas (Nakamura et al., 1984; Diswall et al., 2007), and it can therefore be anticipated that Neu5Gc-terminated glycans are present in all porcine organs. Perhaps with the exception of the brain where Neu5Gc appears to be sparsely expressed (Davies and Varki, 2015).

Neu5Gc linked to GalNAc on *O*-glycans has been identified in pig heart muscle (Jeong et al., 2013). Studies using the anti-HD antibody revealed Neu5Gc-terminating glycolipid compounds in pig hearts and Neu5Gc-GM3 was the most abundant one (Diswall et al., 2010). Several more complex ganglioside species were found but not structurally characterized in detail.

Pig kidneys show strong anti-Neu5Gc staining of all vascular endothelial cells and brush border tubular cells, while the smooth muscle cells of arteries are negative (Reuven et al., 2016). Like the situation in the heart, Neu5Gc-terminating glycolipids were identified in pig kidneys by the anti-HD antibody and Neu5Gc-GM3 was the most abundant one (Diswall et al., 2010). *N*-glycans released from pig kidney cell membrane glycoproteins revealed several novel Neu5Gc-terminated saccharides with up to 14 monosaccharide units present in complex branched structures (Kim et al., 2008). These studies were performed by a combination of HPLC separation of released saccharides followed by MALDI-TOF mass spectrometry. Monosaccharide residues were identified by exoglycosidase digestion (Kim et al., 2008).

ANTI-Neu5Gc ANTIBODIES WITH SPECIAL REFERENCE TO INDUCED ANTI-Neu5Gc ANTIBODIES IN HUMANS EXPOSED TO ANIMAL TISSUE

Hanganutiziu and Deicher (HD) antibodies, the immunodominant group of which is Neu5Gc, were originally identified based on their ability to agglutinate erythrocytes of many animal species (Hanganutziu, 1924; Deicher, 1926). HD antigen-active molecules were later isolated from equine and bovine erythrocytes and were shown to include the Neu5Gc-LacCer (Neu5Gc-GM3) and Neu5Gc-nLc_4Cer glycosphingolipids (Naiki and Higashi, 1980; Mukuria et al., 1986a,b). A glycoprotein from bovine erythrocytes was also shown to be HD antibody-reactive (Naiki and Higashi, 1980; Mukuria et al., 1986a,b). Anti-Neu5Gc antibodies, then defined as HD antibodies, were originally found in sera of patients injected with animal serum but has since then been identified in patients with various malignancies (Malykh et al., 2001) and chronic inflammatory diseases (Padler-Karavani et al., 2013). Whether or not anti-Neu5Gc antibodies are present in the serum of healthy individuals is debated and contradicting results exist in the literature (Mukuria et al., 1986b; Kobayashi et al., 2000; Tangvoranuntakul et al., 2003; Nguyen et al., 2005; Padler-Karavani et al., 2008; Blixt et al., 2009; Huflejt et al., 2009; Le Berre et al., 2017; Leviatan Ben-Arye et al., 2019). To some extent, but perhaps not fully, can these discrepant results be explained by differences in assays and substrates used for their detection (Mukuria et al., 1986b; Kobayashi et al., 2000; Tangvoranuntakul et al., 2003; Nguyen et al., 2005; Padler-Karavani et al., 2008; Blixt et al., 2009; Huflejt et al., 2009). Like blood group ABO (Holgersson et al., 2014), sialyl-Lewis x (Lofling and Holgersson, 2009), and anti-αGal antibodies (McKane et al., 1998) recognize their determinants in a structural context-dependent manner, so do anti-Neu5Gc antibodies (Padler-Karavani et al., 2008). Thus, to detect all Neu5Gc antibodies and not to miss a part of the anti-Neu5Gc repertoire, it is important that the assays used are based on a broad repertoire of Neu5Gc-terminated glycans linked to different core chains and with different linkage configurations between Neu5Gc and the penultimate sugar residue (Padler-Karavani et al., 2008). For this purpose, the glycan microarray and in which antibody reactivity with pairs of Neu5Ac- and Neu5Gc-terminated glycans based on the same core saccharide chain are compared, appears optimal as the differential and preferred reactivity with the Neu5Gc glycan can be directly ascribed to the *N*-glycolyl group (Padler-Karavani et al., 2011, 2012; Leviatan Ben-Arye et al., 2017, 2019; Bashir et al., 2019).

Like other anti-carbohydrate antibodies, anti-Neu5Gc antibodies develop during the first year of life. However, in contrast to for example ABO antibodies that are believed to be induced in response to bacteria carrying A- or B-like determinants in their lipopolysaccharide or capsular polysaccharide, it is hypothesized that anti-Neu5Gc antibodies are induced by commensal/pathogenic, non-typeable *Haemophilus influenzae* which have taken up Neu5Gc from the diet and incorporated it into its cell surface lipooligosaccharide (Taylor et al., 2010). When it comes to the induced immune response to Neu5Gc following, for example grafting of animal cells/tissues or administration of animal/recombinant proteins carrying Neu5Gc-glycans our knowledge is limited.

Immunization of renal allotransplant recipients upon rabbit anti-human thymocyte induction therapy showed an IgG antibody response with an expanded diversity and *de novo* recognition of different anti-Neu5Gc glycans (Amon et al., 2017). Exposure of humans to anti-thymocyte globulin was associated with a shift in the anti-Neu5Gc IgG repertoire and affected the outcome of subsequent renal allografts (Mai et al., 2018). However, repeated injections of recombinant human erythropoietin produced by Chinese hamster ovary cells expressing 1% Neu5Gc did not result in any significant production of anti-Neu5Gc-specific antibodies (Noguchi et al., 1996).

Kobayashi and co-workers studied the anti-Neu5Gc antibody response in patients grafted with fetal porcine pancreatic islets (Groth et al., 1994) and in patients who had their circulation connected to a pig kidney *ex vivo* (Breimer et al., 1996; Rydberg et al., 1996). No significant elevation of IgG and IgM antibody levels against the Neu5Gc-GM3 ganglioside was observed in sera from these patients (Kobayashi et al., 2000). However, the Neu5Gc-GM3 coated ELISA used in this study was later found to be sufficiently sensitive. When individual patients from these clinical trials were tested using a glycan microarray an increase of anti-Neu5Gc antibodies was found in some patients transplanted with pig islets (Blixt et al., 2009). In one of the two patients, who had their circulation connected to a pig kidney, an increase in antibodies binding to Neu5Gc-terminated

GM3 and GD3 gangliosides isolated from pig kidney was found (Magnusson et al., 2003).

Studies of burn patients exposed to live pig skin revealed a statistically significant increase in serum levels of anti-Neu5Gc antibodies in patients compared to controls (Scobie et al., 2013). However, the increase in the mean anti-non-αGal IgG antibody level in the patient group was due to some patients responding, while other patients did not show any increase in anti-non-αGal IgG antibody levels. Blocking studies in selected patients, using Neu5Gc/Neu5Ac, suggested that Neu5Gc glycans were the major non-αGal antigens that induced the antibody response, although other non-αGal antigens might also be involved (Scobie et al., 2013).

Studies on xeno-antibody responses in patients grafted with BHV have been conducted focusing on anti-Gal antibody levels, which were shown to be increased in patients receiving BHVs compared to controls (Konakci et al., 2005; Bloch et al., 2011; Park et al., 2013). So far, no studies investigating anti-Neu5Gc antibody levels following BHV implantation have, to our knowledge, been reported.

In summary, the knowledge regarding the immune response to Neu5Gc glycans in humans exposed to animal tissues is limited as is the knowledge regarding the potential clinical significance of anti-Neu5Gc antibodies in allo- and xenograft rejection.

NOVEL ANTI-Neu5Gc ANTIBODY DIAGNOSTICS SHOULD DETECT AS MUCH AS POSSIBLE OF THE DIVERSE ANTI-Neu5Gc ANTIBODY REPERTOIRE IN A HIGH-THROUGHPUT AND REPRODUCIBLE MANNER

In 1986, Mukuria et al. described an enzyme-linked immunosorbent assay (ELISA) for detection of HD antibodies using flat-bottomed 96-well plates coated with purified Neu5Gc-LacCer (Mukuria et al., 1986b). There was an overall good correlation between the HD antibody reactivity obtained with the ELISA and the horse erythrocyte hemagglutination (HA) test (Mukuria et al., 1986b). However, ∼3% of the sera were negative in the ELISA despite a positive HA suggesting that some anti-Neu5Gc antibodies were not detected in the ELISA (Mukuria et al., 1986b). Using another ELISA format in which polyacrylamide (PAA)-based neoglycoconjugates carrying a single Neu5Gc residue in multiple copies were coated in the wells, most human sera were shown to contain anti-Neu5Gc antibodies (Tangvoranuntakul et al., 2003). Reactivity with the corresponding Neu5Ac-PAA glycoconjugate was used as background control. Using flow cytometry and α-galactosidase-treated porcine RBC as target cells in the absence and presence of 7.5 mM Neu5Gc, 17/20 sera from healthy volunteers were shown to contain anti-Neu5Gc antibodies (Zhu and Hurst, 2002).

Realizing that the anti-Neu5Gc repertoire, like other anti-carbohydrate antibody repertoires, is polyclonal and binds Neu5Gc in different structural contexts determined by the underlying carbohydrate core chain, Padler-Karavani and coworkers developed a novel, innovative ELISA inhibition assay (EIA) aimed at detecting and quantifying a broader portion of the anti-Neu5Gc repertoire (Padler-Karavani et al., 2013). The EIA relied on the difference in reactivity of anti-Neu5Gc antibodies in human serum with WT and *Cmah*-KO mouse serum (Padler-Karavani et al., 2013). To remove all human antibodies reacting with mouse protein and carbohydrate antigens except Neu5Gc, human serum was pre-absorbed on mouse serum from *Cmah*-KO mice and then incubated in wells coated with mouse serum from WT mice. The rationale being that only remaining anti-Neu5Gc antibodies are detected on WT mouse serum. Using this assay, the authors detected an elevated anti-Neu5Gc response in patients with an acute Kawasaki's disease compared to patients with aneurysms or dilated coronary arteries (Padler-Karavani et al., 2013). A potential caveat with this assay is the fact that the proteome and glycome of mouse serum may vary between individuals of the same strain and between the *Cmah*-KO and WT strains even though they are of the same genetic background. Thus, reproducibility over time can be hard to achieve.

Printed glycan microarrays are powerful tools for determining the fine binding specificity of glycan-binding proteins such as carbohydrate-specific antibodies (reviewed in Smith et al., 2010; Rillahan and Paulson, 2011). Arrays directed at determining the fine specificity of sialoside-binding proteins have been developed (Padler-Karavani et al., 2011, 2012; Leviatan Ben-Arye et al., 2017). They have been successfully used to determine the fine specificity of sialoside-binding plant and animal lectins as well as carbohydrate-binding antibodies (Padler-Karavani et al., 2011, 2012; Leviatan Ben-Arye et al., 2017). By printing pairs of Neu5Ac- and Neu5Gc-terminated glycans, the specificity of polyclonal and monoclonal anti-Neu5Gc antibodies have been elucidated (Leviatan Ben-Arye et al., 2017). A high-throughput format of the latter can be used to assess 16 serum samples on one printed slide (Leviatan Ben-Arye et al., 2017). It is important, however, to realize that the chemistries used to produce, present and couple the glycan to the glass slide will all influence the results. Thus, glycan arrays carrying identical glycan structures may not always give similar results (Padler-Karavani et al., 2012; Bashir et al., 2019).

Despite, the very important contributions of glycan arrays to the specificity-determination of anti-Neu5Gc antibody repertoires in health and disease, there is still a need for novel assays allowing quantification of the structurally diverse anti-Neu5Gc repertoire in a reproducible manner and which can be used in clinical routine laboratories on large patient cohorts. Investigations of large patient cohorts suffering from various chronic inflammatory and malignant disorders will be necessary to investigate the full scope of the medical importance of anti-Neu5Gc antibodies.

CONCLUSION

In addition to the αGal antigen determinant, glycans with terminal Neu5Gc residues may constitute an immunogenic barrier for xenografts into humans. However, firm evidence for the role of Neu5Gc antibodies in xenograft rejection is

lacking. Because the immune biology of the anti-Neu5Gc response is slightly different from both the ABO and anti-Gal antibody responses, further studies are needed to better define the exact role of the Neu5Gc antibody repertoire in the xenorejection process. Because carbohydrate antigens are quite resistant to destruction/removal by the procedures used in the manufacturing of bioprosthetic products of animal origin, these antigen determinants must be considered when using live as well as chemically modified animal cells/tissues/organs for treatment of end stage human organ failure. Animals genetically engineered to silence the CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) responsible for the biosynthesis of Neu5Gc have been generated and may be used as source animals for future xenografting including procurement of tissues for bio-prosthesis manufacturing.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Synthesis of *N*-Glycolylneuraminic Acid (Neu5Gc) and Its Glycosides

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Sialic acids constitute a family of negatively charged structurally diverse monosaccharides that are commonly presented on the termini of glycans in higher animals and some microorganisms. In addition to *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolyl neuraminic acid (Neu5Gc) is among the most common sialic acid forms in nature. Nevertheless, unlike most animals, human cells loss the ability to synthesize Neu5Gc although Neu5Gc-containing glycoconjugates have been found on human cancer cells and in various human tissues due to dietary incorporation of Neu5Gc. Some pathogenic bacteria also produce Neu5Ac and the corresponding glycoconjugates but Neu5Gc-producing bacteria have yet to be found. In addition to Neu5Gc, more than 20 Neu5Gc derivatives have been found in non-human vertebrates. To explore the biological roles of Neu5Gc and its naturally occurring derivatives as well as the corresponding glycans and glycoconjugates, various chemical and enzymatic synthetic methods have been developed to obtain a vast array of glycosides containing Neu5Gc and/or its derivatives. Here we provide an overview on various synthetic methods that have been developed. Among these, the application of highly efficient one-pot multienzyme (OPME) sialylation systems in synthesizing compounds containing Neu5Gc and derivatives has been proven as a powerful strategy.

Keywords: sialic acid, sialoside, Neu5Gc, chemical synthesis, chemoenzymatic synthesis

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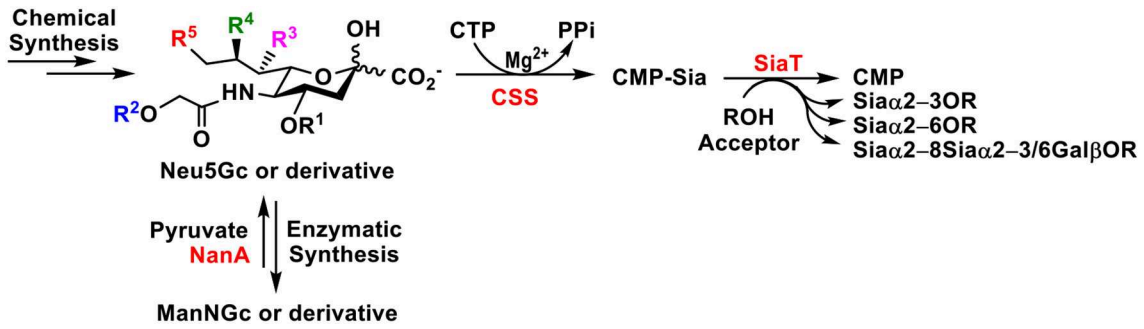
INTRODUCTION

Sialic acids (Sias) are a family of negatively charged monosaccharides with a nine carbon backbone. More than 50 structurally distinct Sias have been found in nature (1–3), out of which more than 15 have been identified in human (4–6). They are commonly presented as the terminal monosaccharides of the carbohydrate moieties of glycoproteins and glycolipids on cell surface of deuterostome animals, in secreted glycans and glycoconjugates including those in the milk of mammals (2, 7–9). Some microorganisms including pathogenic bacteria also produce sialic acid and sialic acid-containing structures (7, 10). Three basic forms of sialic acids are *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and 2-keto-3-deoxynonulosonic acid (Kdn) (**Figure 1A**) (1–3). In nature, sialic acid-containing oligosaccharides and glycoconjugates are formed mainly by sialyltransferase-catalyzed reactions transferring sialic acid from its activated sugar nucleotide, cytidine 5′-monophosphate-sialic acid (CMP-Sia), to suitable acceptors (11) although trans-sialidases have also been used by parasites and bacteria to harvest sialic acids from the hosts to decorate their own surface (12, 13). Neu5Ac is the most common form of sialic acids. Compared to Neu5Ac, Neu5Gc has an extra oxygen, presented as the hydroxyl in the *N*-glycolyl group at C-5.



GRAPHICAL ABSTRACT | Chemical and chemoenzymatic synthetic methods for Neu5Gc and Neu5Gc-sialosides are reviewed. One-pot multienzyme (OPME) chemoenzymatic strategy has advantages in accessing a large number of Neu5Gc-sialosides and their derivatives.

The biosynthesis of Neu5Ac from uridine 5′-diphosphate *N*-acetylglucosamine (UDP-GlcNAc) in eukaryotic cells takes place in the cytosol by three enzymes. The first two committed steps are hydrolytic epimerization of UDP-GlcNAc to form *N*-acetylmannosamine (ManNAc) followed by phosphorylation to form ManNAc-6-P catalyzed by a single bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc-6-kinase (GNE). Phosphoenolpyruvate is then condensed with ManNAc-6-P by Neu5Ac 9-phosphate synthase (NAPS) to produce Neu5Ac-9-P which is dephosphorylated to form Neu5Ac by Neu5Ac-9-phosphate phosphatase (NANP). The Neu5Ac synthesized in the cytosol is transferred into nucleus and used to form CMP-Neu5Ac, the activated form of Neu5Ac, by CMP-sialic acid synthetase (CSS). CMP-Neu5Gc formed in the cytosol from CMP-Neu5Ac by CMP-Neu5Ac hydroxylase (CMAH)-catalyzed reaction (11, 14–17) is transferred into Golgi and used by various sialyltransferases to form glycoconjugates which are secreted or expressed on cell surfaces (**Figure 1B**) (10, 18, 19). The *CMAH* gene is inactive in humans. Therefore, humans do not biosynthesize Neu5Gc-containing structures themselves (20, 21). New World monkeys were also shown to loss the function of Neu5Gc production due to an independent CMAH inactivation (22).

Regardless of CMAH inactivation in human, Neu5Gc has been found on the cell surface of human tumors and even in normal human tissues although at a lower amount (23, 24). Neu5Gc in human glycoconjugates comes likely from the consumption of animal-derived diets, such as red meat and animal milk (25–27). On the other hand, during infancy (around the age of 6 months) humans develop varying levels of polyclonal antibodies of IgG (28, 29), IgM, and IgA (30, 31) types against a diverse array of Neu5Gc-containing glycans (32–35). The mechanism of developing such anti-Neu5Gc antibodies early in the human life is unclear although incorporating dietary Neu5Gc by bacteria colonized in humans, such as non-typeable *Haemophilus influenzae* (NTHi) to form Neu5Gc-containing epitopes, such as cell surface lipooligosaccharides (LOS) is a likely source of the corresponding immunogens (32, 36, 37). So far, *de novo* synthesis of Neu5Gc and Neu5Gc-containing structures has not been demonstrated in bacteria. The presence of *CMAH*-like sequences has been found in the genomes of some bacteria but the activities of the corresponding enzymes have not been confirmed (38–40).

The presence of Neu5Gc-containing xeno-auto-antigens and anti-Neu5Gc xeno-autoantibodies in human (24) may lead to potential complications, such as chronic inflammation namely "xenosialitis" (41), atherosclerotic cardiovascular diseases, cancers, and autoimmune diseases (34, 38, 42–45). In addition, exposure to clinically used Neu5Gc-presenting animal-derived biotherapeutics (such as immunosuppressant rabbit anti-human thymocyte globulin, ATG) elicited anti-Neu5Gc antibodies (46, 47) with a profile that may be different from the "pre-existing" ones (28, 48). The biological consequences of this have not been revealed. A recent analysis showed that treating kidney transplant patients with ATG did not increase the risk of colon cancer (49). Neu5Gc has also been found on biodevices (such as bioprosthetic heart valves) which may affect their duration of function due to interaction with anti-Neu5Gc antibodies which can lead to calcification (50, 51). Furthermore, Neu5Gc in addition to α-Gal epitopes presented on animal tissues causes barriers for animal-to-human xenotransplantation (such as porcine skin xenografting and organ xenotransplantation) (52, 53).

Neu5Gc and its glycosides are important tools for profiling anti-Neu5Gc antibodies and sialic acid-binding proteins, understanding Neu5Gc-related immune responses, and designing potential therapeutics. To better understand their important roles, it is critical to obtain structurally defined glycans and glycoconjugates containing Neu5Gc or its derivatives.

Neu5Gc and derivatives have been found and can be isolated from natural sources including non-human mammals, some higher invertebrates, such as sea urchin, sea cucumber, and starfish (11, 23, 54, 55), as well as the surface of salmonid fish eggs (56). For example, Neu5Gc has been extracted from sea cucumber *Cucumaria echinate* in 99% purity. It constitutes about 85% of the total sialic acids in dry weight of Gumi (sea cucumber), and 23.6 mg was obtained from 135 g of fresh body weight (57). Neu5Gc-containing oligosaccharides have been reported in the milk of primates, domestic herbivores, pigs, lion, and leopard (58). So far, twenty-two Neu5Gc derivatives (**Figure 2**) have been reported (1, 3). These include mono-, di-,

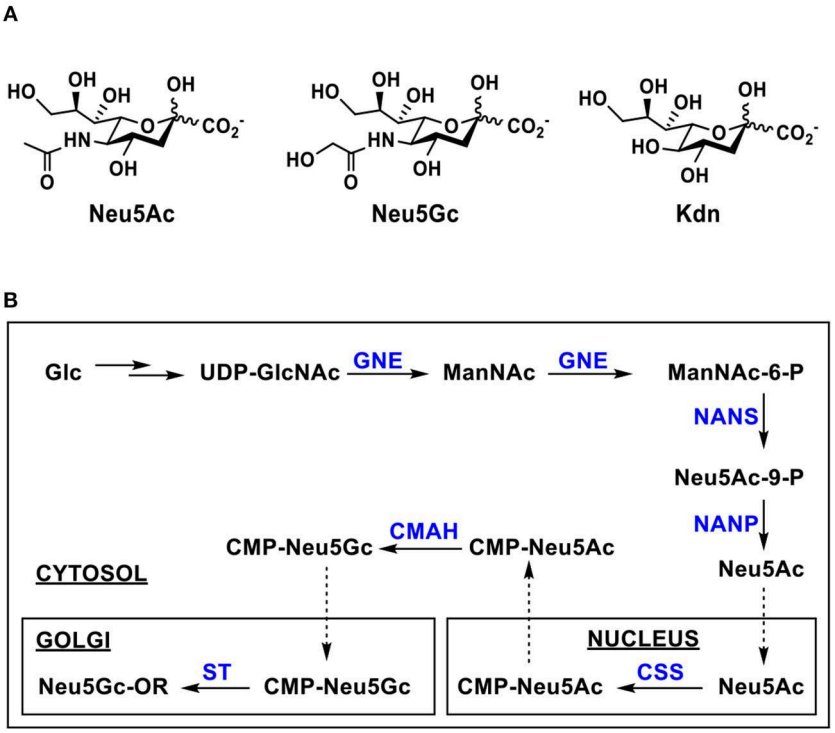


FIGURE 1 | (A) Three basic forms of sialic acids including *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (Kdn); **(B)** Biosynthesis of Neu5Gc and its sialosides in eukaryotic cells. Enzymes and abbreviations: GNE, UDP-GlcNAc 2-epimerase/ManNAc-6-kinase; NANS, Neu5Ac-9-P synthetase; NANP, Neu5Ac-9-P phosphatase; CSS, CMP-sialic acid synthetase; CMAH, cytidine 5′-monophosphate-N-acetylneuraminic acid hydroxylase.

and tri-*O*-acetylation at C4, C5, C7, C8, and/or C9 positions in Neu5Gc as well as other modifications including *O*-methylation at C5- or C8-, *O*-lactylation at C9, or *O*-sulfation at C8 or C9 of Neu5Gc with or without *O*-acetylation. Neu5Gc1,7lactone has also been identified.

Neu5Gc and derivatives can link to other carbohydrate moieties with different sialyl linkages including α2–3- and α2–6-linked to galactose; α2–6-linked to *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose or glucose; α2–8- and α2–9-linked to another Sia molecule; and α2–5-linked between polymers of Neu5Gc (7, 10, 59–61), adding diversity to sialic acid-containing compounds. The modification and linkage patterns of Sia play a pivotal role in many biochemical processes, such as cell signaling, cell-cell interaction, cellular adhesion, inflammation, fertilization, viral infection and malignancies, and regulation of apoptosis and proliferation (62, 63).

Numerous outstanding reports have been published describing the synthesis of sialic acids and sialoside. The focus, however, has been on Neu5Ac-containing compounds. The synthesis of Neu5Gc-containing glycans is attracting an increasing attention in recent years. This review provides an overview of various chemical and chemoenzymatic synthetic methods developed for the production of Neu5Gc and derivatives as well as the corresponding sialosides.

CHEMICAL AND CHEMOENZYMATIC SYNTHESIS OF Neu5Gc AND DERIVATIVES

Only a limited number of naturally occurring and non-natural Neu5Gc derivatives have been chemically or chemoenzymatically synthesized.

Neu5Gc was chemically synthesized from D-arabinose by the Wong group. The C5-acylamino group of Neu5Gc and a vinyl group were simultaneously introduced to D-arabinose by a modified Petasis coupling reaction. The vinyl group was then converted to γ-hydroxy-α-keto acid by a 1,3-dipolar cycloaddition reaction with *N*-*tert*-butyl nitrone followed by a base-catalyzed β elimination and hydrolysis to produce Neu5Gc in 22% overall yield (64).

O-Acetylation is the most frequent modification of Neu5Gc in nature. 9-*O*-Acetyl-Neu5Gc (Neu5Gc9Ac) has been found in bovine submandibular gland glycoprotein (65, 66). On the other hand, 4-*O*-acetyl-Neu5Gc (Neu4Ac5Gc) has been found in horse glycoproteins (61), α2–8-linked polysialic acids on glycoproteins from unfertilized kokanee salmon egg (67), the serum of guinea pigs (68), and gangliosides in human colon cancer tissues (69). Both Neu5Gc9Ac and Neu4Ac5Gc have been successfully synthesized. The use of orthoester intermediates is a very efficient method for producing

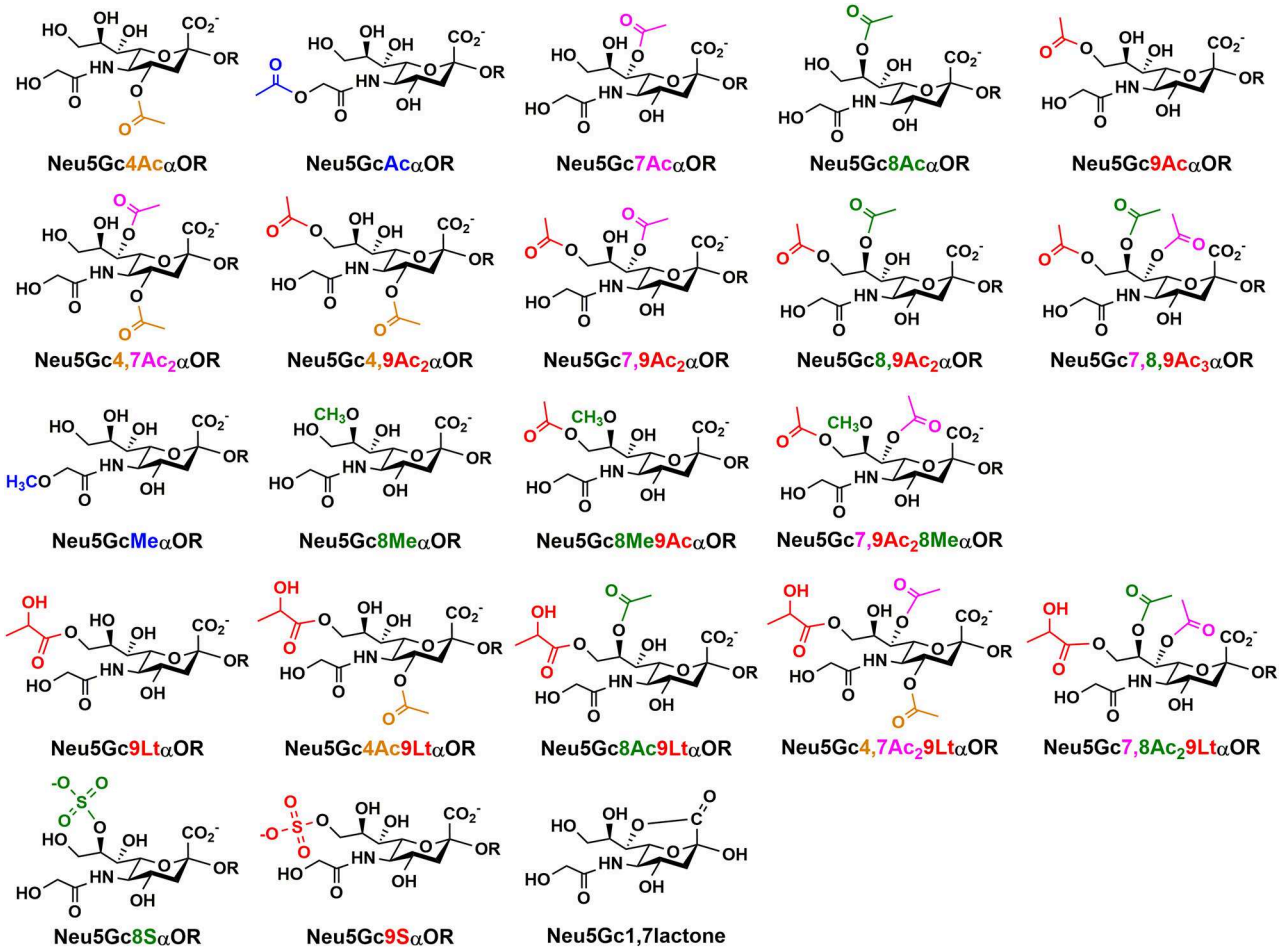


FIGURE 2 | Naturally occurring Neu5Gc derivatives that have been identified.

various 9-*O*-acyl derivatives of Neu5Gc. Highly regioselective acylation at C9-hydroxyl of Neu5Gc was achieved by the treatment of Neu5Gc with a trimethyl orthoacetate in the presence of a catalytic amount of *p*-toluenesulfonic acid (*p*-TsOH) to form Neu5Gc9Ac in 90% yield. A similar strategy was applied for the synthesis of non-natural derivatives of Neu5Gc including 9-*O*-butyroyl-Neu5Gc and 9-*O*-benzoyl-Neu5Gc in 88 and 70% yields, respectively (70). On the other hand, Neu4Ac5Gc was synthesized using an efficient chemoenzymatic approach involving a sialic acid aldolase-catalyzed reaction from D-mannosamine ($ManNH_2$) acylated with a benzyl protected *N*-glycolyl group. The obtained *N*-(2-benzyloxyacetyl)-D-mannosamine was enzymatically converted to a Neu5Gc derivative in a quantitative yield by recombinant *Pasteurella multocida* sialic acid aldolase (PmNanA) (71). Following a number of selective protection strategies, 4-hydroxyl group was selectively acetylated. The desired 4-OAc-Neu5Gc was obtained in an overall yield of 46% after de-protection of other hydroxyl groups (72).

In nature, the major function of sialic acid aldolases is to break down sialic acids, such as Neu5Ac to form 6-carbon amino sugar *N*-acetylmannosamine (ManNAc) and a three-carbon metabolite pyruvic acid. Nevertheless, they are capable of catalyzing the reversed reaction and have been used as synthetically useful enzymes for the formation of sialic acids and derivatives. Sialic acid aldolase-catalyzed reactions can be a general and highly efficient approach for chemoenzymatic synthesis of a diverse array of Neu5Gc and derivatives from the corresponding *N*-glycolylmannosamine (ManNGc) and derivatives. PmNanA was found to have a better expression level and more promiscuous substrate specificity than the more commonly used *Escherichia coli* sialic acid aldolase (EcNanA) in catalyzing the formation of sialic acids and derivatives (71). Both enzymes have been used for chemoenzymatic synthesis of Neu5Gc and derivatives. For the synthesis of Neu5Gc from ManNGc by sialic acid aldolase-catalyzed reaction, ManNGc could be obtained by chemical synthesis from D-mannosamine ($ManNH_2$) (73, 74) or D-glucose (75), or by alkaline epimerization of *N*-acetylglucosamine (GlcNAc) (76). For the synthesis of ManNGc from $ManNH_2$, the *N*-glycol group could be installed using commercially available inexpensive acetoxyacetyl chloride followed by de-*O*-acetylation by hydrolysis under a basic

condition. However, it was found that ManNGc could be epimerized to form *N*-glycolylglucosamine (GlcNGc) under even mild basic conditions. *Aspergillus niger* lipase (Amano A) was found to be efficient in de-*O*-acetylation without the problem of epimerization (76). Installing the *N*-glycol group using *N*-succinimidyl glycolate (74) or 2-(benzyloxy)acetyl chloride followed by hydrogenation (77) could also avoid the complication of epimerization. The Neu5Gc formed went through additional chemical reactions for the synthesis of *N*-glycolyl-2,3-dehydro-2-deoxyneuraminic acid (Neu5Gc2en) (78), a transition state analog inhibitor of some sialidases. Together with *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid (Neu5Ac2en), they have been found to be effective in protecting mice from bacteria sepsis in a CD24/SiglecG-dependent manner (79) in a cecal ligation and puncture (CLP) mouse model (80). The protection was improved by combining the use of Neu5Ac2en and Neu5Gc2en with antibiotic treatment (79). In addition, Neu5Gc2en alone was effective in protecting mice from endotoxemia by inhibiting mouse sialidase NEU1 expressed on cell surface upon lipopolysaccharide (LPS) stimulation (81).

The sialic acid aldolase-catalyzed reactions can also be used to synthesize naturally occurring and non-natural derivatives of Neu5Gc. As shown in **Figure 3A**, Neu5Gc derivatives with C5 and/or C9-modifications have been synthesized by sialic acid aldolase-catalyzed reactions from C2- and/or C6-substituted ManNGc derivatives as their 6-carbon sugar precursors (74, 82–87). Naturally occurring 8-*O*-methyl Neu5Gc (Neu5Gc8Me) (**Figure 3B**) was also synthesized from chemically synthesized 5-*O*-methyl ManNGc (ManNGc5Me) by a PmNanA-catalyzed reaction. A good yield of 86% was achieved using five equivalents of sodium pyruvate in Tris-HCl buffer (100 mM, pH 7.5) at 37°C for 24 h followed by the combination of anion exchange chromatography and gel filtration column purification (88).

EcNanA-catalyzed aldol addition of ManNGc and 3-fluoro-pyruvate resulted in a mixture of 3F(*equatorial*)Neu5Gc and 3F(*axial*)Neu5Gc with a ratio of close to 1:1. They were readily separated by a simple flash chromatography (**Figure 3C**) (89).

Disaccharides with a ManNGc at the reducing end could also be suitable substrates for EcNanA. Two chemically synthesized disaccharides Galα1–2ManNGc and Galβ1–2ManNGc were used as the substrates for EcNanA for the synthesis of the corresponding disaccharides Galα1–5Neu5Gc and Galβ1–5Neu5Gc in 36 and 34% yields, respectively (**Figure 3D**) (85).

SYNTHESIS OF SIMPLE GLYCOSIDES OF Neu5Gc

Simple glycosides of Neu5Gc have been synthesized from the corresponding Neu5Ac derivatives by directly de-*N*-acetylating the *N*-acetyl group of Neu5Ac under a strong basic condition followed by acylation and deprotection. For example, as shown in **Figure 4A**, the *N*-acetyl group in the carboxyl protected allyl α-Neu5Ac-glycoside was removed to produce the free amino group in 80% yield by refluxing in tetramethylammonium hydroxide. Acylation with acetoxyacetyl chloride followed by hydrolysis of the ester produced the desired allyl α-Neu5Gc-glycoside (90). An improved microwave-assisted de-*N*-acetylation process was also reported (91). In this case, fully protected methyl α-Neu5Ac glycoside was treated with 2.0 M of NaOH under an optimized microwave irradiation condition (15 min at 120°C at a maximum power of 100 W) produced the desired 5-amino derivative in 91% yield. The resulting compound was then converted to the target methyl α-Neu5Gc glycoside (Neu5GcαOMe) by reacting with acetoxyacetyl chloride, followed by de-*O*-acetylation (**Figure 4B**). The same method was applied successfully for the formation of Neu5Gc2en from per-acetylated Neu5Ac2en methyl carboxylate as well as the production of poly-Neu5Gc from the corresponding α2–8-linked homopolymer of Neu5Ac (91).

An 9-azido derivative of Neu5Gc2en (Neu5Gc9$N_3$2en) was also chemically synthesized from Neu5Ac9$N_3$2en by substituting the 9-hydroxyl group with an azido group followed by replacing the -NHAc moiety with *N*-glycolyl group (92).

An alternative strategy for the synthesis of Neu5GcαOMe (**Figure 4C**) involved enzymatic formation of Neu5Gc from ManNGc using an EcNanA-catalyzed reaction. Protection of Neu5Gc, followed by activation, glycosylation, and deprotection led to the formation of the desired Neu5GcαOMe which was used for ELISA inhibition assays and for purifying anti-Neu5Gc antibodies from human sera (33).

CHEMICAL SYNTHESIS OF Neu5Gc-CONTAINING OLIGOSACCHARIDES

Several chemical glycosylation methods have been developed for the synthesis of Neu5Gc-containing oligosaccharides. The following discussion will be focused on different types of glycosyl donors used.

Glycosyl Chloride Donors

A glycosyl chloride donor was used for synthesizing Neu5Acα2–5Neu5Gc disaccharide which contained a sialyl α2–5-Neu5Gc linkage similar to that found in the poly(-5Neu5Gcα2-) structure on the jelly coat of sea urchin eggs (93). The strategy involved the formation of an allyl glycoside of protected Neu5Ac as an important intermediate which went through oxidative cleavage of the C=C double bond in the allyl group (94) to form a protected Neu5Ac glycoside with a carboxymethoxy aglycone. Most recently, a similar strategy using protected Neu5Gc allyl glycoside donor was applied in the synthesis of Neu5Gcα2–5Neu5Gc disaccharide building block for the formation of a tetrasaccharide capped with 9-*O*-sulfo-Neu5Gc (Neu5Gc9S) found on sea urchin egg surface proteins (95).

The same Neu5Ac glycosyl chloride donor was used for glycosylation with methyl glycolate. The glycosylated product was deprotected and de-*N*-acetylated to form an amino-containing intermediate which can be either protected by a fluorenylmethyloxycarbony (Fmoc) group at the amino group or by a methyl group on the carboxyl groups. The resulting compounds were coupled to form the amide bond, linking two sialic acid units together to produce the desired

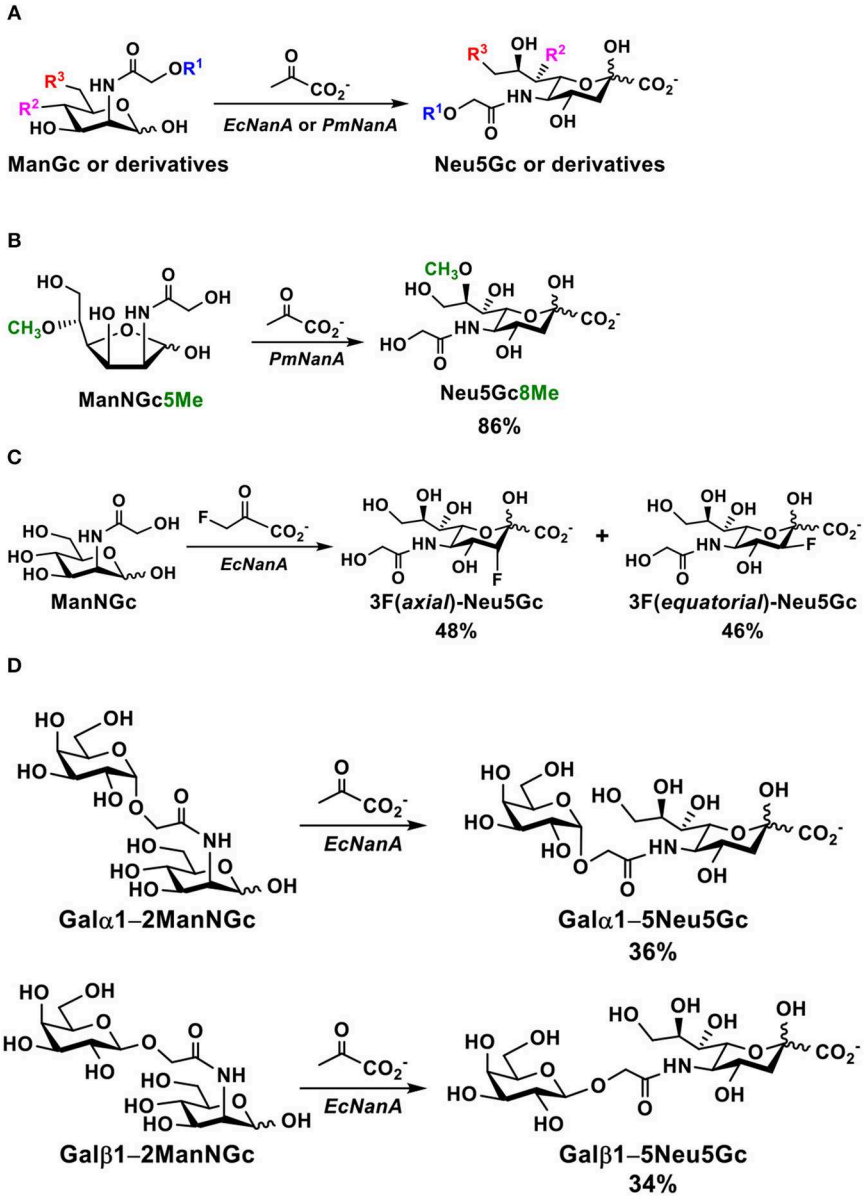


FIGURE 3 | (A) A general chemoenzymatic synthetic strategy of sialic acid aldolase (EcNanA or PmNanA)-catalyzed synthesis of Neu5Gc and derivatives containing modifications at C5, C7, and/or C9 from ManNGc and derivatives, **(B)** PmNanA-catalyzed synthesis of Neu5Gc8Me, **(C)** EcNanA-catalyzed synthesis of 3-fluoro-Neu5Gc, and **(D)** EcNanA-catalyzed synthesis of disaccharides containing Neu5Gc at the reducing end.

disaccharide containing a Neu5Gc residue (96). α2–5-Linked Neu5Gc oligomers for up to octasaccharide were also synthesized using a similar strategy by coupling carboxyl and amine protecting groups of sialic acid building blocks by amide formation (97).

An *O*-acetyl protected Neu5Gc glycosyl chloride donor was also used for the synthesis of Neu5Gcα2–3Galβ1–4Glc trisaccharide building block for the formation of Neu5Gc-GM3 ganglioside although with a low yield and a poor stereo-selectivity (98).

Thioglycoside Donors

Sialyl thioglycoside donors have been widely applied in chemically formation of sialyl glycosidic bonds. A thioglycoside donor of Neu5Ac was used for the synthesis of Neu5Acα2–5Neu5Gc disaccharide found as the structural component of the jelly coat of sea urchin eggs. The strategy relied on the formation of a protected Neu5Ac glycoside with a carboxymethoxy aglycone which was readily coupled with the amino group of the protected neuraminic acid to form the desired amide bond in the disaccharide (93).

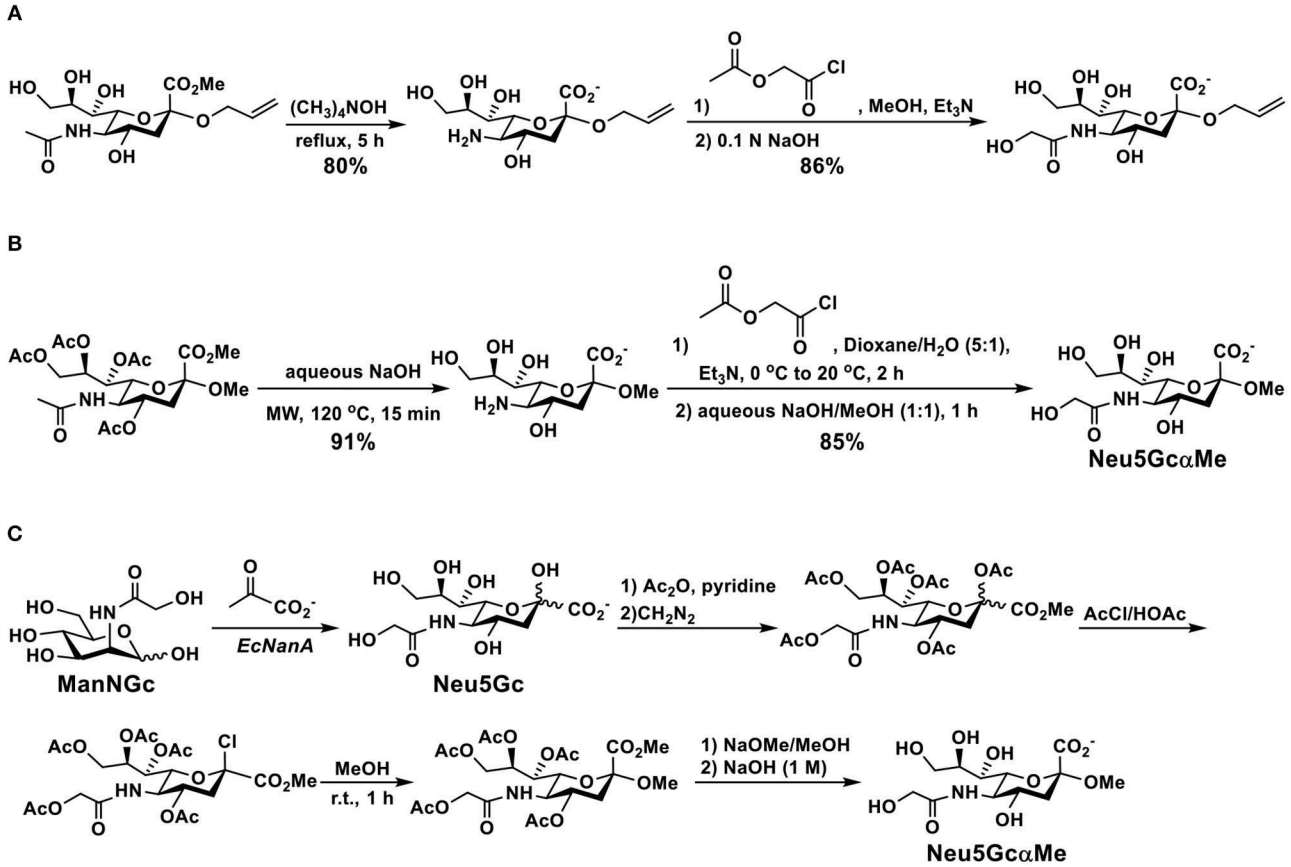


FIGURE 4 | Synthesis of Neu5Gc-glycosides by de-*N*-acetylation of Neu5Ac-glycoside derivatives without **(A)** or with **(B)** a microwave-assisted process and **(C)** by glycosylation of protected Neu5Gc formed enzymatically from ManNGc and pyruvate.

Thioglycoside donors of Neu5Gc have also been used for the synthesis of more complex Neu5Gc-containing sialosides. The Kiso group reported the synthesis of protected Neu5Gcα2–3GalβOMP disaccharide using *N*-2,2,2-trichloroethoxycarbonyl (Troc)-protected thiophenyl sialoside donor which was readily obtained from its corresponding *N*-acetyl derivative. Sialylation of a selectively protected galactoside acceptor led to the formation of sialyl disaccharide. Removal of the *N*-Troc group by zinc in acetic acid formed a free amino group which can be acylated with acetoxyacetyl chloride to produce the desired protected Neu5Gc-containing disaccharide (99). A similar strategy was used for the synthesis of a Neu5Gc8Me-containing tetrasaccharide building block of the pentasaccharide component, Neu5Gc8Meα2–3(Neu5Gc8Meα2–6)GalNAcβ1–3Galβ1–4Glc, in GAA-7 ganglioside (100). In addition to the use of acetoxyacetyl chloride as a reagent for introducing a protected glycolyl group to the amino group on neuraminic acid (Neu) residue for the formation of Neu5Gc, 1,3-dioxolan-2,4-dione (101) prepared from glycolic acid was also used for the formation of Neu5Gc-GM1 ganglioside directly from naturally more abundant Neu5Ac version of GM1 (102).

The Sato group used a *N*-Troc-protected thiophenyl sialoside donor for the synthesis of sialyllactoside component of ganglioside LL3 tetrasaccharide. The removal of the *N*-Troc group followed by conjugation with a protected Neu5Ac glycoside with a carboxymethoxy aglycone and deprotection steps formed the desired LL3 tetrasaccharide (103, 104).

The amino intermediate of the protected sialoside formed after the removal of the *N*-Troc group could be converted directly (99) to a 1,5-lactamized bicycle structure. Alternatively, *N*-trifluoroacetyl (*N*-TFA)-protected thiophenyl sialoside donor can also be used similarly for the formation of sialyl glycosides. The *N*-TFA group could be readily removed and the resulting amino group-containing intermediate could be converted to a 1,5-lactamized bicycle structure under mild basic conditions. The resulting intermediate could be selectively protected at C-9 of the sialic acid and used as a well-suited sialylation acceptor. A similar *N*-Troc and 8,9-acetal-protected thiotoluene sialoside donor was used for the synthesis of protected Neuα2–6GalαSer as the sialyl Tn disaccharide building block that was coupled with the pre-formed Neu5Gcα2–5Neu5Gc disaccharide component for the formation of the sea urchin egg surface Neu5Gc9S-capped tetrasaccharide (95).

Trisaccharide Neu5Gcα2–4Neu5Acα2–6Glc, a structural component of ganglioside HLG-2, was synthesized by the Kiso group by stereoselective coupling of *N*-Troc-protected

thiophenyl Neu5Gc-sialoside donor with the pre-formed Siaα2–6Glc 1,5-lactamized disaccharide acceptor (105, 106). A similar strategy was used for the synthesis of Fucα1–4Neu5Acα2–5Neu5Gcα2–4Neu5Acα2–6Glc, a pentasaccharide component of HPG-7 ganglioside (107) and Fucα1–8Neu5Gcα2–4Neu5Acα2–6Glc, a tetrasaccharide components of ganglioside HPG-1 (108).

The Crich group reported the synthesis of Neu5Gc-containing oligosaccharides in high stereoselectivity by iterative one-pot route. A series of four trisaccharides were synthesized in one pot by coupling of a 5*N*-acetoxyacetimide-5*N*, 4*O*-oxazolidinone-protected adamantanyl thiosialoside donor with the first thiogalactosyl acceptor followed by addition of the second acceptor after 20 min (109).

The Nifant'ev group used an *N*-*tert*-butyloxycarbonyl (*N*-Boc) and *N*-acetyl (*N*-Ac) protected thiophenyl sialoside donor for the synthesis of 3-aminopropyl glycoside of Neu5Gcα2–6LacNAc from *N*-acetyllactosamine (LacNAc) 4′,6′-diol acceptor (30). A glycosylation yield of 84% with 1.3:1 (α:β) selectivity was achieved. Removal of the *N*-acetyl and *N*-Boc groups followed by *N*-acylation and subsequent deprotection steps formed the desired trisaccharide. A similar strategy was used for the synthesis of 3-aminopropyl glycoside of Neu5Gcα2–3LacNAc using LacNAc 2′,3′,4′-triol acceptor (110).

Instead of installing *N*-glycolyl group after the formation of sialyl glycosidic bond, properly protected Neu5Gc thioglycoside donors could be directly used for glycosylation. For example, an acetyl-protected thiophenyl Neu5Gc-glycoside donor was used directly with α-selectivity and good sialylation yields for the synthesis of Neu5Gc-containing glycosides including sialyl Lewis × pentasaccharyl ganglioside analog (111) and α2–3-sialyl lactotetraose and neolactotetraose derivatives (112). A thiophenyl Neu5Gc-glycoside donor was also successfully used for the synthesis of Neu5Gcα2–6GalOMP disaccharide and its derivative Neu5Gc9N_3α2–6GalOMP containing a 9-azido-9-deoxy-Neu5Gc residue. The 9-azido group of the latter was converted to an amino group and the resulting compound was used to generate a library of 9-*N*-acylated derivatives of Neu5Gc-sialosides. Some of the compounds were low-micromolar inhibitors of CD22 (or Siglec-2), a well-known B cell-specific sialic acid-binding immunoglobulin-like lectin (113). The same strategy was used to synthesize a similar class of sialosides with different aglycons as improved CD22 inhibitors with up to nanomolar potency (114, 115). In addition to protected thiophenyl Neu5Gc-glycoside donors, a benzyl-protected thiomethyl Neu5Gc-glycoside donor was developed and used for the synthesis of Neu5Gc-containing trisaccharides with 55–63% yields with α-selectivity (116) and a sea cucumber disaccharyl ganglioside analog (117).

Phosphite Donors

Phosphite donors of Neu5Gc are considered to be more reactive than thioglycoside donors. They were used for the synthesis of Neu5Gc-glycosides in propionitrile at −78°C in good yields and α-selectivity (**Figure 5**) (118).

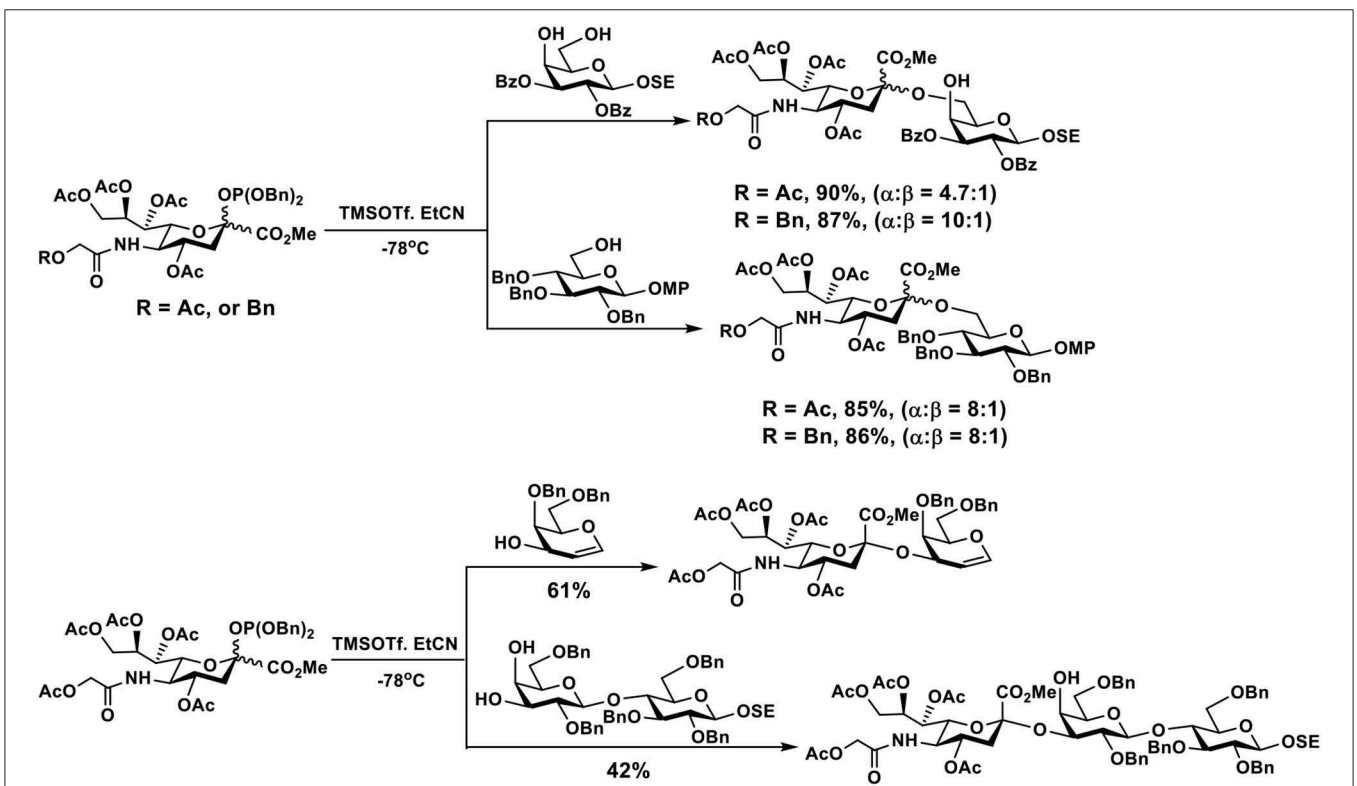


FIGURE 5 | Efficient synthesis of Neu5Gc-glycosides using phosphite donors in good yields and α-selectivity.

Trichloroacetimidate Donors

Trichloroacetimidate donors are the most commonly used glycosyl donors. They have been used for the synthesis of complex Neu5Gc-containing sialosides. The Kiso group reported the first total synthesis of Neu5Gc8Me-containing ganglioside GAA-7 which showed neuritogenic activity. The strategy involved the assembly of the ceramide moiety by Witting, Grignard, and amide formation reactions. Stereoselective β-glycosylation with a glucosyl trichloroacetimidate donor produced a glucosyl ceramide (Glcβ1Cer) cassette which was readily coupled with the protected Neu5Gc-containing tetrasaccharyl trichloroacetimidate donor to form the protected ganglioside. Global deprotection produced GAA-7, a pentasaccharyl β-ceramide Neu5Gc8Meα2–3(Neu5Gc8Meα2–6)GalNAcβ1–3Galβ1–4Glcβ1Cer (119). Protected Neu5Gc-containing disaccharyl trichloroacetimidate donors have also been used for the synthesis of Neu5Gc-containing glycans of lacto- and neolacto-series gangliosides. The reducing ends of these oligosaccharides were further modified by 2-(tetradecyl)hexadecanol to form glycolipid mimics of ceramide-containing gangliosides (120).

N-Phenyltrifluoroacetimidate Donors

N-Phenyltrifluoroacetimidate (121) sialyl donors were designed to improve their reactivity for glycosylation. The feature was combined with 5-*N*-phthaloyl group protection of the sialyl donors to favor α-sialyl isomer formation (122, 123) and allow their suitability for one-pot procedures (124). As shown in **Figure 6**, desired α-sialoside was stereoselectively synthesized using this donor. The synthesized α-sialoside was further coupled to another acceptor in one-pot to synthesize trisaccharides with various internal disaccharide units. The 5-*N*-phthaloyl group on sialic acid of trisaccharides was readily removed, acylated, and deprotected to form the *N*-glycolyl group in Neu5Gc (124).

CHEMOENZYMATIC SYNTHESIS OF Neu5Gc-CONTAINING OLIGOSACCHARIDES

Sialyltransferase-catalyzed glycosylation can be considered as the most efficient approach for the production of sialic acid-containing structures. The strategy offers great advantages, including high regioselectivity and stereoselectivity for the formation of sialyl linkages as well as mild reaction condition in aqueous solutions, etc. (2, 10). The increasing availability of substrate promiscuous sialyltransferases in large amounts makes the strategy practical even for large-scale synthesis. As the sugar nucleotide donor, CMP-Neu5Gc, for sialyltransferase-catalyzed synthesis of Neu5Gc-glycosides is not commercially available, additional enzymes including CMP-sialic acid synthetases (CSSs) with or without sialic acid aldolases are commonly used. Although biosynthetically CMP-Neu5Gc is directly synthesized from CMP-Neu5Ac by CMAH-catalyzed hydroxylation, Neu5Gc is a well-tolerated substrate for CSSs from bacterial sources including those from *Neisseria meningitidis* (NmCSS), *Escherichia coli* (EcCSS), *Streptococcus agalactiae* serotype V (SaVCSS), *Pasteurella multocida* strain P-1059 (PmCSS), *Haemophillus ducreyi* (HdCSS), and *Clostridium thermocellum* (CtCSS) (74, 125, 126). Among these, NmCSS with a high expression level, a high specific activity, and substrate promiscuity is an excellent choice for chemoenzymatic synthesis of sialosides with or without sialic acid modifications (125).

Starting from pyruvate and a mixture of ManNGc and GlcNGc, chemoenzymatic synthesis of trisaccharide Neu5Gcα2–3Galβ1–3GalNAc, which has been found in porcine submaxillary mucin, was achieved (127). As shown in **Figure 7**, Neu5Gc was synthesized in 59% yield using an immobilized sialic acid aldolase. It was used for the formation of CMP-Neu5Gc using an immobilized calf brain CMP-sialic acid synthetase in 60% yield. Sialylation of Galβ1–3GalNAcβOBn was carried out by a porcine liver α2–3-sialyltransferase-catalyzed reaction using CMP-Neu5Gc as donor. Deprotection by catalytic hydrogenation produced the target trisaccharide Neu5Gcα2–3Galβ1–3GalNAc in 56% yield.

As reaction conditions for sialic acid aldolase, CSS, and sialyltransferase are compatible, they can be mixed together in one-pot with ManNGc, pyruvate, CTP, and a sialyltransferase acceptor for the synthesis of target Neu5Gc-glycosides. Such one-pot multienzyme (OPME) sialylation reactions (82, 84, 86, 128) are highly efficient for chemoenzymatic synthesis of a large library of Neu5Gc-glycosides containing different sialyl linkages and various internal glycans. Sialosides containing modified Neu5Gc forms can also be produced by this strategy.

As shown in **Figure 8A**, in the OPME reaction containing a sialic acid aldolase, a CSS, and a sialyltransferase, chemically synthesized ManNGc or derivative is enzymatically converted to Neu5Gc or derivative by the sialic acid aldolase. Activation of the formed Neu5Gc or derivative to CMP-Neu5Gc or derivative by CSS followed by sialylation led to the production of the desired sialoside containing Neu5Gc or derivative. Both sialic acid and CMP-sialic acid are generated *in situ* and do not need to be purified.

If Neu5Gc and derivatives are available, OPME reaction containing a CSS and a sialyltransferase without the presence of a sialic acid aldolase (**Figure 8B**) is sufficient to produce sialosides containing Neu5Gc or derivatives. The strategy is particularly suited for sialosides containing a Neu5Gc derivative that cannot be directly obtained by a sialic acid aldolase-catalyzed reaction, such as Neu4Ac5Gc (72). The method was also used for synthesizing sialosides containing 3F(*equatorial*)-Neu5Gc or 3F(*axial*)-Neu5Gc. In this case, 3F(*equatorial*)-Neu5Gc, and 3F(*axial*)-Neu5Gc were pre-synthesized from ManNGc and 3-fluoro-pyruvate by EcNanA-catalyzed reaction and purified before being subjected to OPME sialylation reactions.

Using efficient OPME sialyltransferase systems with two- or three-enzymes (**Figure 8**), a diverse array of sialosides including glycosphingolipid glycans, sialylated types 1–5 glycans, and sialyl Tn, containing Neu5Gc (**Table 1**) as well as sialosides containing different Neu5Gc derivatives including 3F-Neu5Gc, Neu4Ac5Gc, Neu5Gc9Ac, Neu5GcMe, or Neu5GcAc (**Table 2**) have been synthesized. The obtained compounds have been used to construct sialyl glycan microarrays (28, 33, 34, 42, 83, 150–156), sialoside-protein

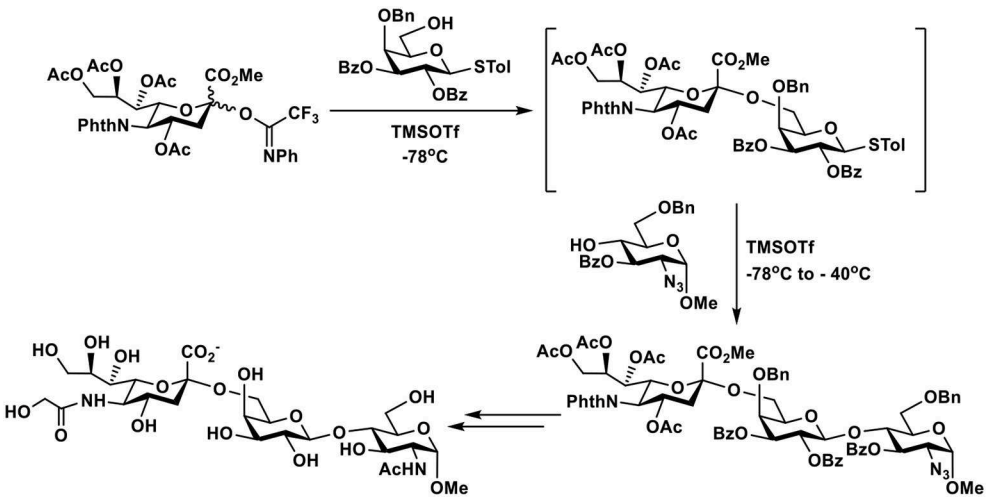


FIGURE 6 | An example of one-pot chemical synthesis of Neu5Gc-containing trisaccharides using 5-*N*-phthaloyl group protected *N*-phenyltrifluoroacetimidate sialyl donor.

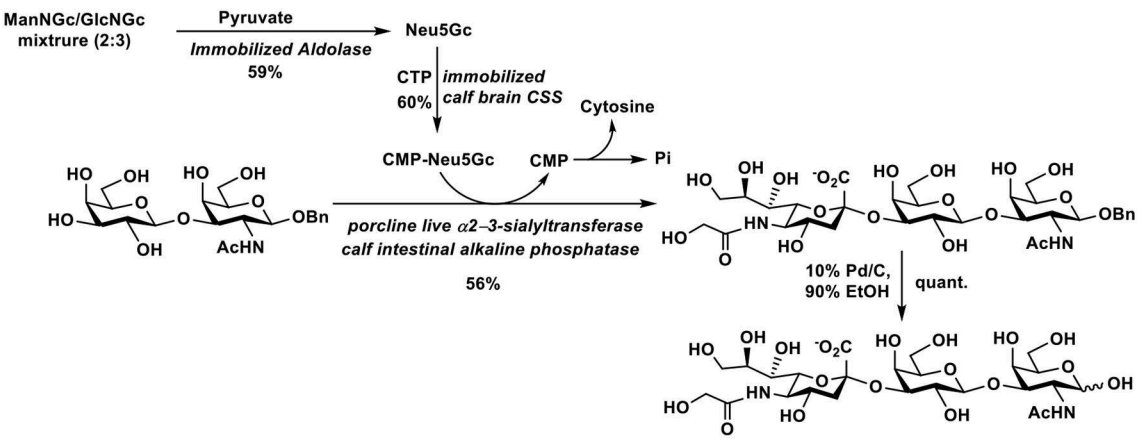


FIGURE 7 | Chemoenzymatic synthesis of Neu5Gcα2–3Galβ1–3GalNAc.

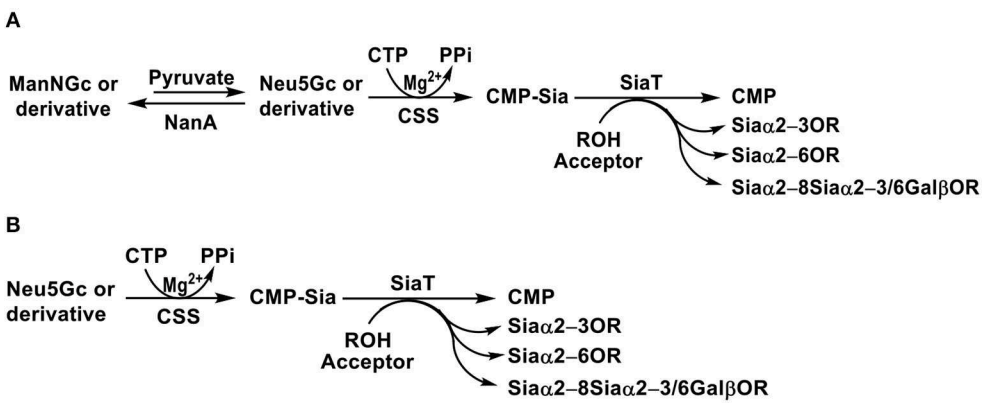
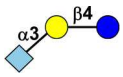
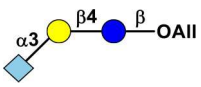
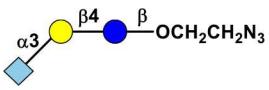
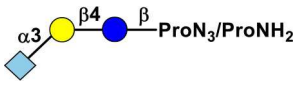
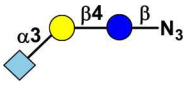
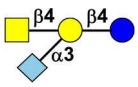
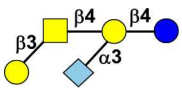
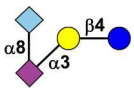
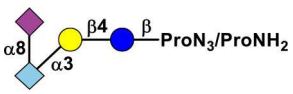
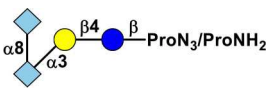
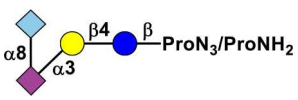
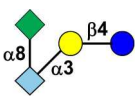
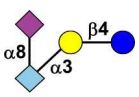
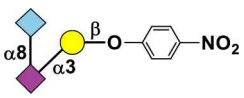
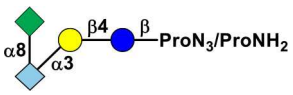
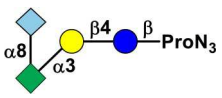
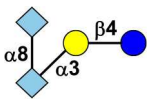
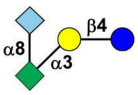
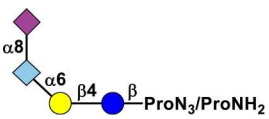


FIGURE 8 | Synthesis of sialosides containing Neu5Gc or derivative using one-pot multienzyme (OPME) sialylation systems containing **(A)** three enzymes including sialic acid aldolase (NanA), CMP-sialic acid synthetase (CSS), and sialyltransferase (SiaT) or **(B)** two enzymes including CSS and SiaT.

TABLE 1 | Chemoenzymatically synthesized Neu5Gc-containing glycans.

Neu5Gc-glycosides	References	Neu5Gc-glycosides	References	Neu5Gc-glycosides	References
Ganglio-series					
GM3 type glycans					
	(77, 127)		(129)		(130)
	(29, 84)		(84)		
GM2 type glycans					
	(51)				
GM1 type glycans					
	(77)				
GD3 type glycans					
	(77)		(29, 86)		(29, 86)
	(29, 86)		(77)		(77)
	(131)		(29, 86)		(86)
	(77)		(77)		(29, 86)
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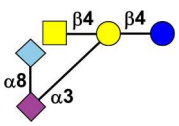
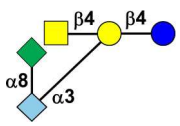
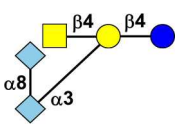
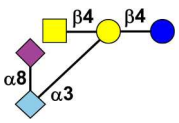
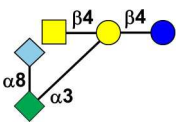
	(77)		(77)		(77)
	(77)		(77)		
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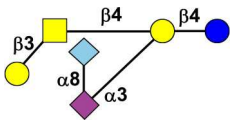
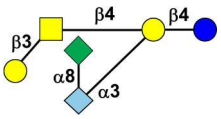
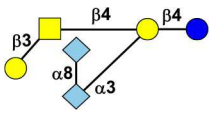
	(77)		(77)		(77)

(Continued)

TABLE 1 | Continued

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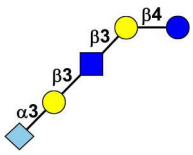
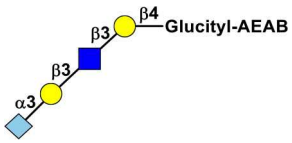
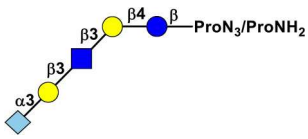
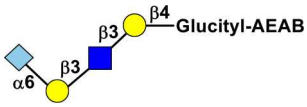
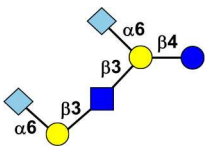
	(77)		(83)		(29, 132, 133)
	(83)		(134)		
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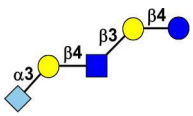
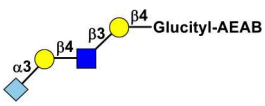
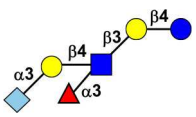
	(77)		(83)		(77)
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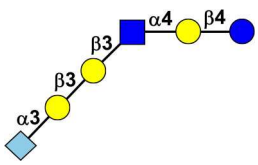
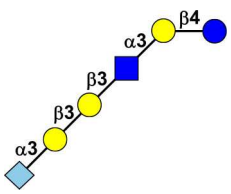
	(77)		(77)		
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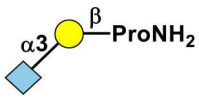
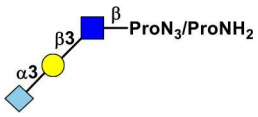
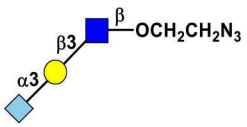
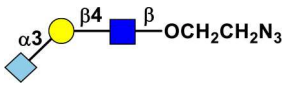
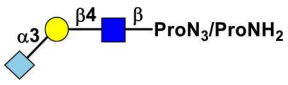
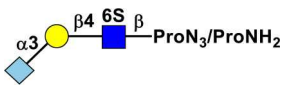
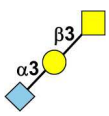
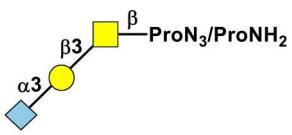
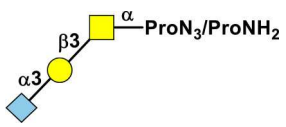
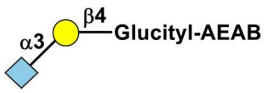
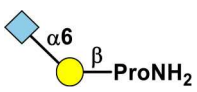
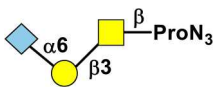
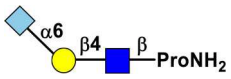
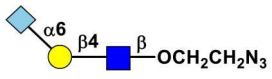
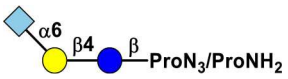
	(29)		(29, 132)		(130, 135)
	(130, 135)		(29, 132)		(29, 132)
	(127)		(29, 132)		(29, 132, 136)
	(83)		(29)		(137)
	(29)		(130, 135)		(29, 82)

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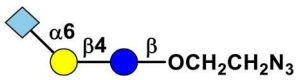
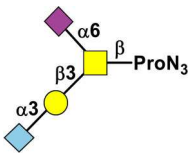
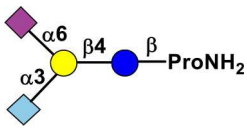
TABLE 1 | Continued

Neu5Gc-glycosides	References	Neu5Gc-glycosides	References	Neu5Gc-glycosides	References
	(130, 135)		(83)		(138)
	(29)				
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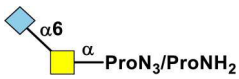
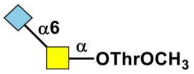
	(29, 87)		(130, 135, 139)		
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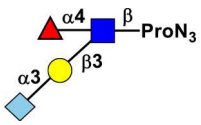	(140)				
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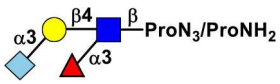
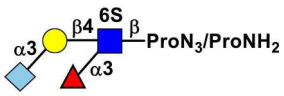
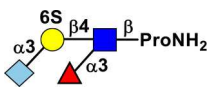
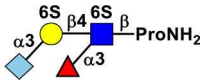
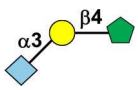
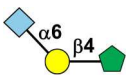
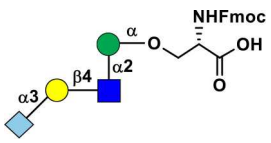
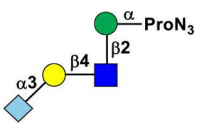
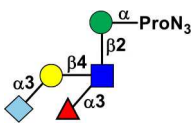
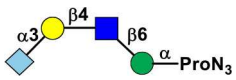
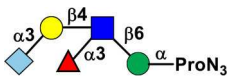
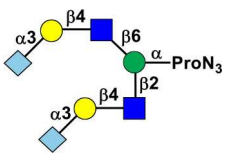
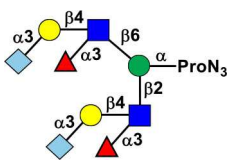
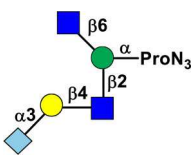
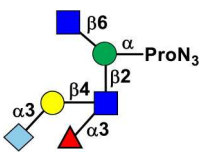
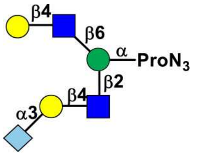
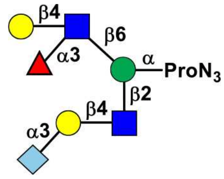
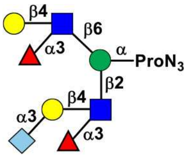
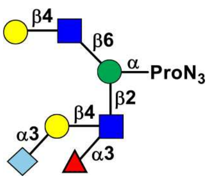
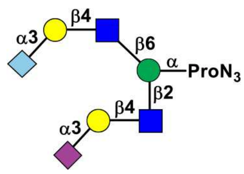
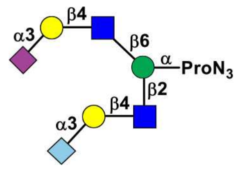
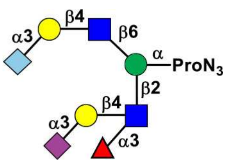
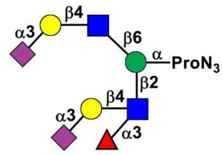
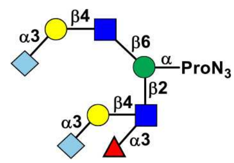
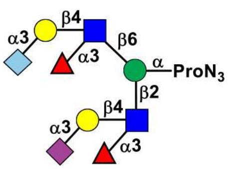
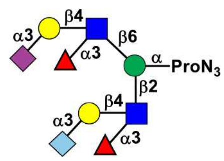
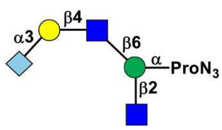
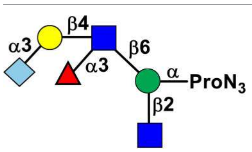
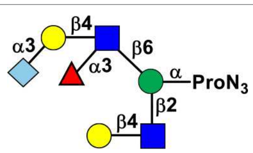
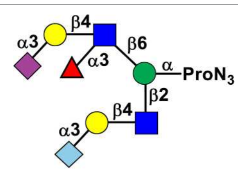
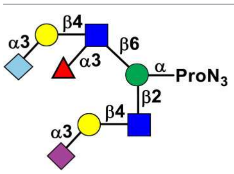
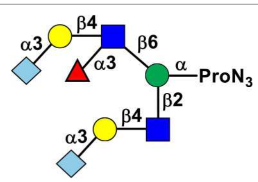
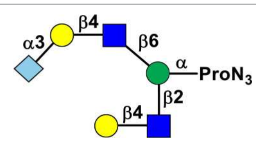
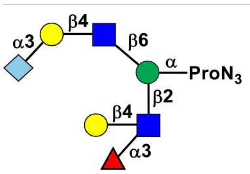
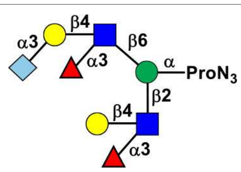
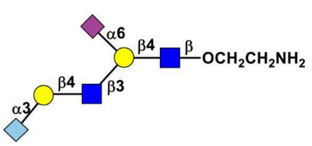
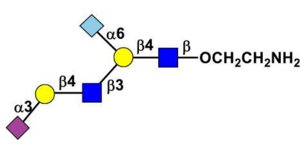
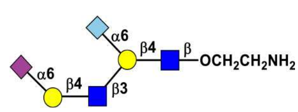
	(29, 141)		(29, 142)		(142)
	(142)				
Sialyl Lactuloses					
	(143)		(143)		
Mammalian O-Mannose glycans					
	(144)		(145)		(145)
	(145)		(145)		(145)
	(145)		(145)		(145)

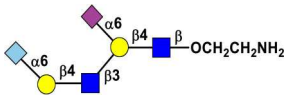
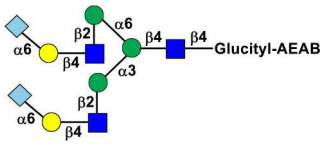
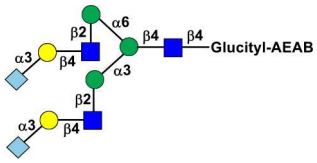
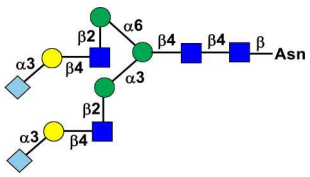
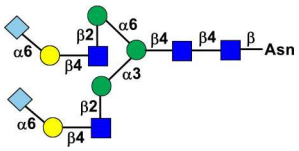
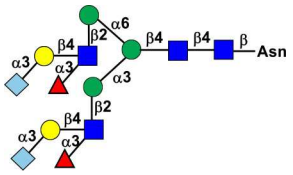
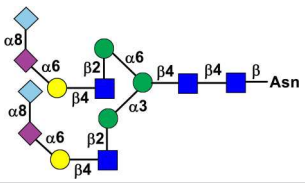
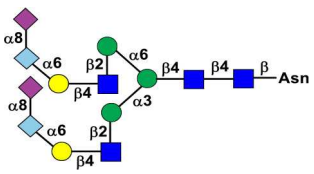
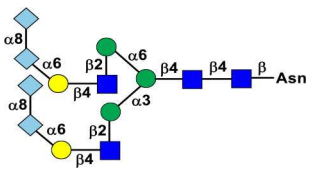
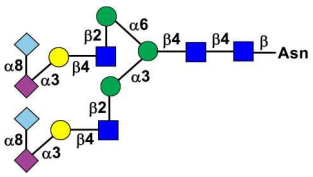
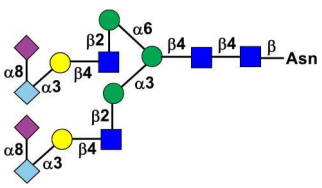
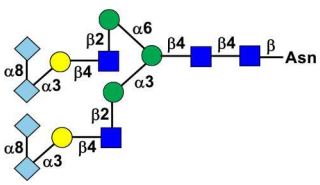
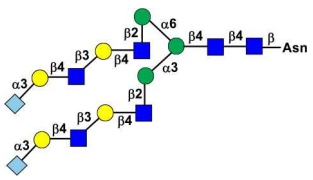
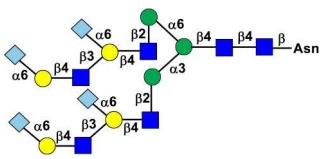
(Continued)

TABLE 1 | Continued

Neu5Gc-glycosides	References	Neu5Gc-glycosides	References	Neu5Gc-glycosides	References
	(145)		(145)		(145)
	(145)		(145)		(145)
	(145)		(145)		(145)
	(145)		(145)		(145)
	(145)		(145)		(145)
	(145)		(145)		(145)
	(145)		(145)		
Sialylated Poly-Lacnac					
	(146)		(146)		(146)

(Continued)

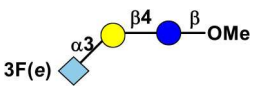
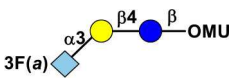
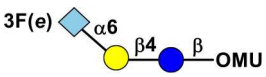
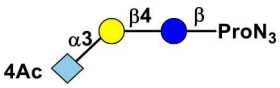
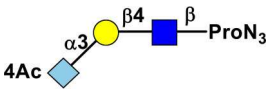
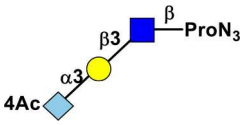
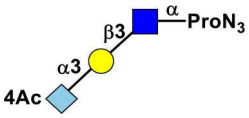
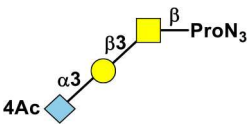
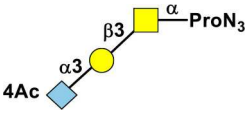
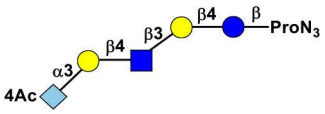
TABLE 1 | Continued

Neu5Gc-glycosides	References	Neu5Gc-glycosides	References	Neu5Gc-glycosides	References
	(146)				
***N*-Glycans**					
	(83)		(83)		(147)
	(147)		(147)		(147)
	(147)		(147)		(147)
	(147)		(147)		(147)
	(146, 147)				


Glc, Gal, Neu5Ac, Neu5Gc, Kdn, GalNAc, Fuc, GlcNAc, Man, Fruc. All, allyl group; Me, methyl; ProN$_3$, propyl azide; ProNH$_2$, propyl amine; Glucityl-AEAB, the reductive amination product of glucose and 2-amino-N-(2-aminoethyl)-benzamide.

conjugates (148), and sialidase substrate specificity studies (131, 157–159). Among bacterial sialyltransferases used, *Pasteurella multocida* sialyltransferase 1 (PmST1) (84) and its single mutant PmST1 M144D with decreased donor hydrolysis and sialidase activities (141) were broadly applied for the synthesis α2–3-linked sialyl oligosaccharides containing Neu5Gc, 3F-Neu5Gc, Neu5Gc9Ac, Neu5GcMe, or Neu5GcAc (77, 83, 84, 89, 157, 160). For synthesizing α2–3-sialyl oligosaccharides containing Neu4Ac5Gc, however, only *Pasteurella multocida* sialyltransferase 3 (PmST3) (161) was found to be a suitable enzyme (72). PmST3 was also well-suited for the synthesis of α2–3-linked Neu5Gc-containing sialyl glycopeptides (162). *Photobacterium damselae* α2–6-sialyltransferase (Pd2,6ST) (82), *Photobacterium* species α2–6-sialyltransferase (Psp2,6ST) (87) and its single mutant with improved expression level and slightly enhanced activity Psp2,6ST A366G (163) were used for synthesizing α2–6-linked sialosides containing Neu5Gc, 3F-Neu5Gc, Neu5Gc9Ac, Neu5GcMe, or Neu5GcAc (82, 83, 89, 157, 160). Psp2,6ST was well-suited for the synthesis of sialyl Tn-antigens (Sia α2–6GalNAcαOR) (87). *Campylobacter jejuni* sialyltransferase CstII (CjCstII) (164) was found to be an efficient sialyltransferase for the synthesis of a diverse array of Neu5Gc-containing α2–8-linked sialosides (77, 86, 131). For synthesizing sialosides containing Neu5Gc or its stable analogs, such as 3F-Neu5Gc and Neu5GcMe, the pH of the OPME reactions was controlled at

TABLE 2 | Chemoenzymatically synthesized sialosides containing 3FNeu5Gc, Neu4Ac5Gc, Neu5Gc9Ac, Neu5GcMe, Neu5GcAc, or Neu5GcBn.

Sialosides	References	Sialosides	References	Sialosides	References
3FNeu5Gc-containing glycans					
	(63)		(63)		(63)
Neu4Ac5Gc-containing glycans					
	(72)		(72)		(72)
	(72)		(72)		(72)
	(72)				
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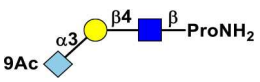
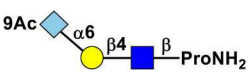
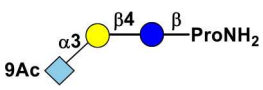
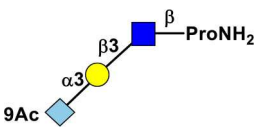
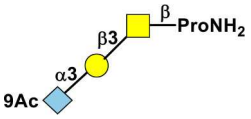
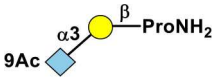
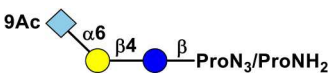
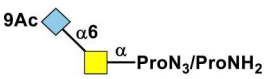
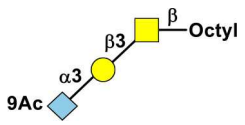
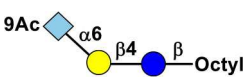
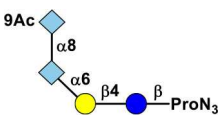
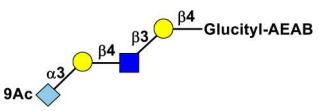
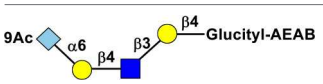
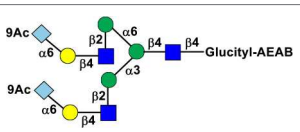
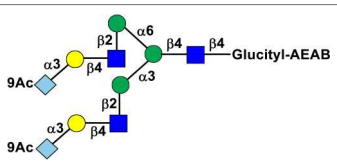
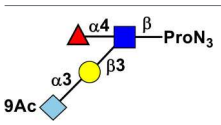
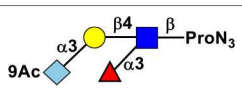
	(128)		(128)		(128)
	(128)		(128)		(128)
	(29, 82, 148)		(29, 148)		(149)
	(149)		(86)		(83)
	(83)		(83)		(83)
	(140)		(141)		

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TABLE 2 | Continued

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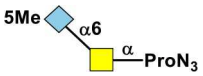
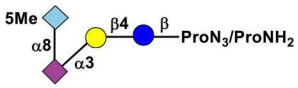
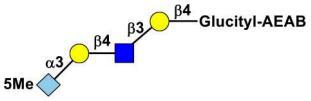
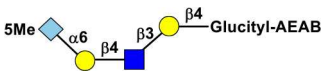
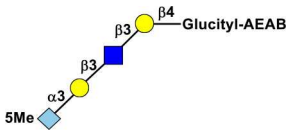
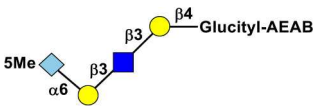
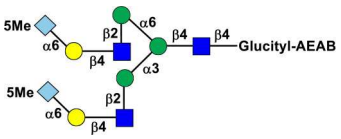
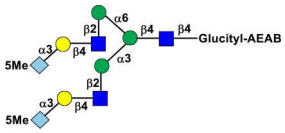
	(87)		(29, 86)		(83)
	(83)		(83)		(83)
	(83)		(83)		
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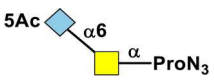
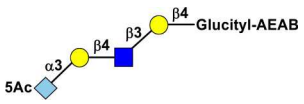
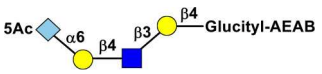
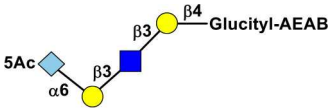
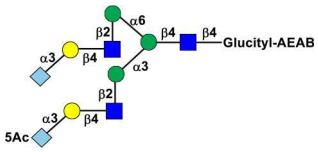
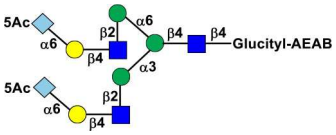
	(87)		(83)		(83)
	(83)		(83)		(83)
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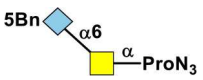	(87)				

Glc, Gal, Neu5Ac, Neu5Gc, Fuc, GalNAc, Man, GlcNAc. Me, methyl; ProN$_3$, propyl azide; ProNH$_2$, propyl amine; Glucityl-AEAB, the reductive amination product of glucose and 2-amino-N-(2-aminoethyl)-benzamide.

8.5 to allow highly efficient catalysis by all enzymes involved in the reactions. For synthesizing sialosides containing base-labile groups, such as Neu4Ac5Gc, Neu5GcAc, or Neu5Gc9Ac, the pH of the OPME reactions was controlled at 7.0 to minimize de-*O*-acetylation during the reaction.

Recently, the OPME α2–3-sialylation system containing PmNanA, NmCSS, and PmST1 M144D was coupled with *Streptococcus pneumoniae* sialidase SpNanC-catalyzed reaction for the formation of Neu5Gc2en from ManNGc, pyruvate, CTP, and lactose (165).

CHEMOENZYMATIC SYNTHESIS OF Neu5Gc-CONTAINING GLYCOCONJUGATES

The alkyl azido aglycone in chemoenzymatically synthesized Neu5Gc-containing sialosides can be readily converted to an alkyl amino group by catalytic hydrogenation to allow convenient conjugation with *N*-hydroxysuccinimide-activated or epoxide-activated slide surface for generating glycan microarrays (34). It was also used to react with adipic acid *p*-nitrophenyl diester to form half-esters which were coupled to the amino group (e.g., in lysine residues) of biotinylated human (STn) antigens Neu5Gc/Neu5Gc9Acα2–6GalNAcαOR (**Figure 9A**) and sialyl lactosides Neu5Gcα2–6Galβ1–4GlcβOR (**Figure 9B**) containing Neu5Gc or Neu5Gc9Ac were successfully synthesized and used for ELISA inhibition studies (33, 148).

OPME chemoenzymatic sialylation reactions have also been used in the synthesis of sialyl-Tn-MUC1 and sialyl-T-MUC1 glycopeptides containing Neu5Gc (**Figure 9C**). *Pasteurella multocida* α2–3-sialyltransferase (PmST3), *Photobacterium damselae* α2–6-sialyltransferase (Pd2,6ST) *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS) and *E. coli* sialic acid

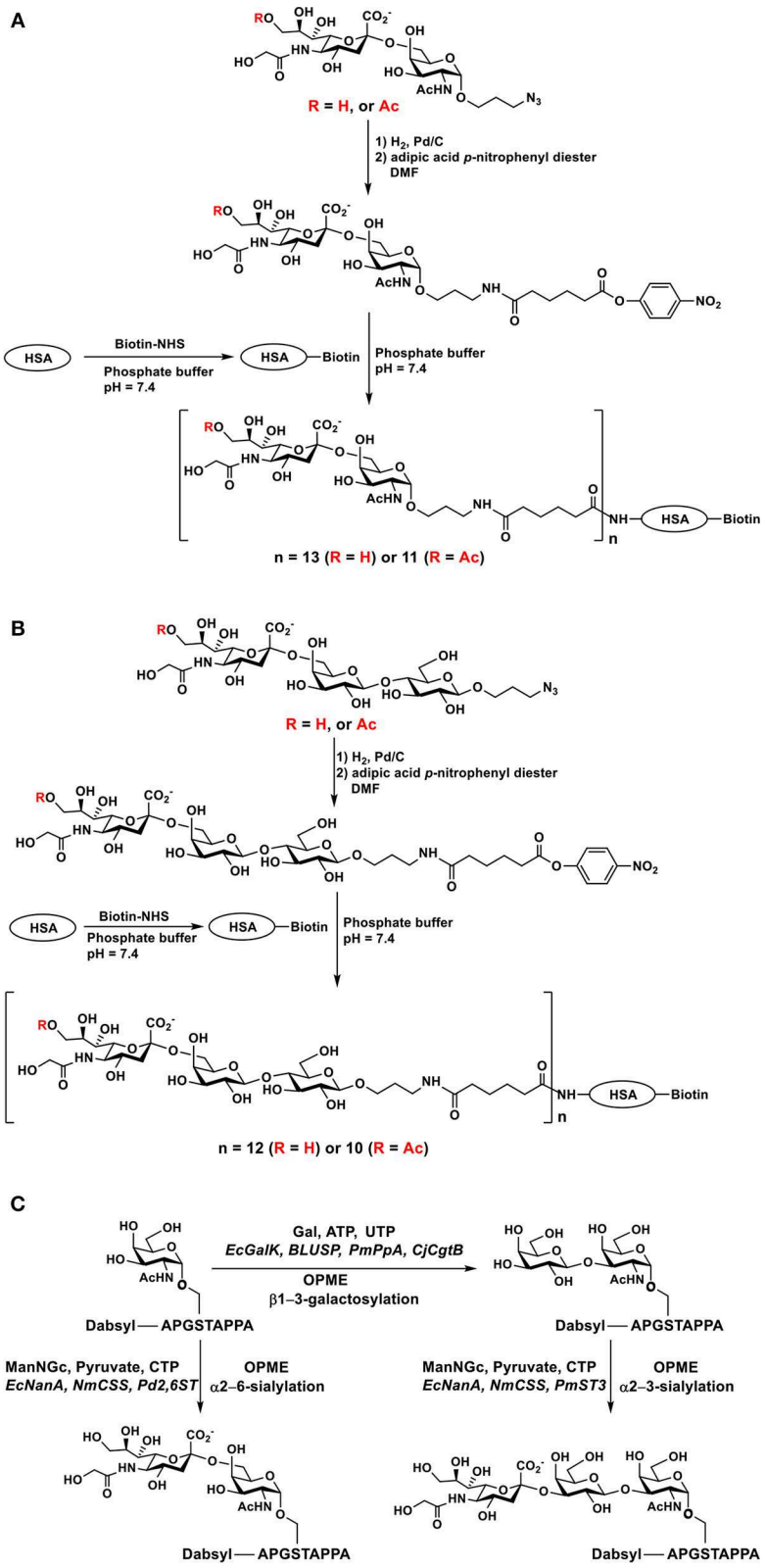

FIGURE 9 | Synthesis of biotinylated human serum albumin-sialoglycoside conjugates containing Neu5Gc or Neu5Gc9Ac including **(A)** sTn epitopes, **(B)** sialyl lactoside, and **(C)** chemoenzymatic synthesis of dabsyl fluorophore-tagged glycopeptides including sTn, T, and ST-antigens containing Neu5Gc. Adopted and modified from Yu et al. (148) and Malekan et al. (162) with permission.

aldolase are the enzymes used for OPME sialylation of glycoproteins (162).

Hidari et al. recently reported the synthesis of multivalent Neu5Gc-containing sialoglycopolypeptides. Treating the chemically synthesized Lac or LacNAc-carrying peptides as acceptors and CMP-Neu5Gc as the donor substrate, sialoglycopolypeptides with α2–3- and α2–6-sialyl linkages were obtained in the presence of ST3Gal III or ST6Gal I, respectively. They found that multivalent α2–3-linked Neu5Gc-ligands selectively inhibited hemagglutination mediated by influenza viruses with a strong inhibitory activity (166). Hernaiz et al. also reported that the enzymatic approach could be directly applied to sialylating lactose-carrying glycoclusters using α2–6-sialyltransferase from rat liver and CMP-Neu5Gc as the donor to produce Neu5Gc-containing glycoclusters (167).

CONCLUSIONS AND PERSPECTIVE

Significant advances have been made in the synthesis of sialosides although the focus has been on those containing Neu5Ac, the most common sialic acid form. With the increasing recognition of the presence and the important functions of Neu5Gc and human anti-Neu5Gc xeno-autoantibodies, more attention has been and will be paid to the synthesis of sialosides containing Neu5Gc and its derivatives. Chemical synthetic methods developed for the formation of Neu5Ac-containing molecules can be extended to Neu5Gc counterparts with modifications. Chemoenzymatic methods using sialyltransferases have been recognized as efficient strategies for accessing challenging sialic acid-containing molecules including those containing Neu5Gc and derivatives. Among these, one-pot multienzyme (OPME) systems have been proven powerful tools. Large library of sialosides containing Neu5Gc and derivatives will become available for elucidating their biological roles and exploring their potential applications. These will be indispensable probes for profiling anti-Neu5Gc antibodies and investigating other Neu5Gc-binding proteins. Such information will help us to better understand the physiological and pathological roles of Neu5Gc and its binding partners. Combining sialidase-treatment and sialyltransferase-catalyzed re-sialylation with Neu5Gc or Neu5Ac will be a potentially efficient approach for generating glycoconjugates with a desired sialic acid form for improved therapeutic applications.

AUTHOR CONTRIBUTIONS

AK, HY, and XC searched the literature, read the papers, and wrote the manuscript.

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Natural and Synthetic Sialylated Glycan Microarrays and Their Applications

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This focused chapter serves as a short survey of glycan microarrays that are available with sialylated glycans, including both defined and shotgun arrays, their generation, and their utility in studying differential binding interactions to sialylated compounds, highlighting N-glycolyl (Gc) modified sialylated compounds. A brief discussion of binding interactions by lectins, antibodies, and viruses, and their relevance that have been observed with sialylated glycan microarrays is presented, as well as a discussion of cross-comparisons of array platforms and efforts to centralize and standardize the glycan microarray data.

Keywords: sialylated glycans, glycan microarrays, synthetic glycans, natural glycans, glycan binding proteins, functional glycomics, Neu5Ac, Neu5Gc

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INTRODUCTION TO GLYCAN MICROARRAYS AS A TOOL TO STUDY GLYCAN BINDING INTERACTIONS

Glycan microarray technology has enabled unprecedented examination of the binding interactions between glycan binding proteins (GBPs) and host carbohydrates, as discoveries are continually being made about the significance and utility of glycans in various biological interactions. The microarray tools can be broadly grouped into (a) defined arrays with known glycan structures, and (b) undefined arrays with unknown structures, which would include arrays derived from natural source material and chemical or enzymatically modified materials that may not be fully characterized. Many of the available glycan microarray platforms contain a variety of sialylated glycans, including the N-acetyl (Ac) and N-glycolyl (Gc) forms. Sialylated glycans have been especially recognized for their significant biological roles, thus creating particular interest in sialylated glycan microarrays.

DIVERSITY OF GLYCAN MICROARRAY PLATFORMS

Synthesis of Sialosides

The sialosides and sialic acid derivatives that populate various microarrays are generated from different sources. Chemo-enzymatic and semi-synthetic approaches have been taken for many compounds, when root glycan structures were available for further modification by sialyltransferases or chemical approaches (Blixt et al., 2004). The most extensive variety of sialosides, containing Ac and Gc forms as well as other modifications, for use on microarrays and in other applications has been derived by the laboratory of Xi Chen (Yu et al., 2005, 2006a,b, 2011; Chokhawala et al., 2008; Linman et al., 2012; Khedri et al., 2014; Li et al., 2017). This work uses novel one-pot synthesis and combinatorial methods, and is the subject of another review in this issue (Chen et al., 2019). The diversity and biological applications of large libraries of natural and non-natural sialosides and their chemical approaches were well described in previous reviews (Deng et al., 2013; Liang et al., 2015).

Consortium for Functional Glycomics

One of the most successful and widely used glycan microarrays has been created by the Consortium for Functional Glycomics (CFG, www.functionalglycomics.org). It is a defined, synthetic glycan microarray and is the largest publicly available glycan microarray with ∼600 printed glycans on the latest version, of which approximately 20 percent are sialylated (**Table 1**; Blixt et al., 2004). There are 14 compounds that contain a Neu5Gc, in either an O-glycan backbone or in a ganglioside structure, as well as the monosaccharide. The CFG array has been extensively utilized to identify and study the interactions of many GBPs including galectins, C-type lectins, and siglecs; immune molecules such as anti-glycan and anti-microbial antibodies and receptors; pathogenic toxins and pathogens such as HIV and parainfluenza virus hemagglutinin-neuraminidase glycoprotein (http://www.functionalglycomics.org/static/consortium/Library.shtml) (Stowell et al., 2008; Hickey et al., 2010; Alymova et al., 2012, 2016; Blackler et al., 2016; Hussein et al., 2016; Jobling, 2016; Jones et al., 2016; Malik et al., 2016; Petrova et al., 2016; Schroeder et al., 2016; Vainauskas et al., 2016; Collins et al., 2017; Noach et al., 2017). The CFG glycan microarray was instrumental in identifying the anti-carbohydrate antibodies found in human intravenous immunoglobulin (IVIG) (Schneider et al., 2015) and helping to elucidate the specificity for Neu5Gc of an *E. coli* derived subtilase toxin (Day et al., 2017).

In addition to mapping the broad recognition of GBPs, the utility of the CFG glycan microarray lies in its versatility for multiple types of comparative binding studies. The arrays can be used to compare sample "sets," as in wild-type vs. mutant proteins or pathogens. Additionally, assay conditions for the same sample can be compared in order to reveal environmental features of the binding interaction such as calcium dependence. While each array is considered an independent experiment, the "relative" binding feature allows for comparisons to be made, and all of the experiments, scanning, and data analysis are performed in a common location to decrease other variables. The glycans found on the array have been chemo/enzymatically produced, and often structures are replicated with one difference, for example in the terminal sialic acid linkage or even the linker molecule, allowing for fine specificity comparisons of what was once thought erroneously to be "minor" differences. As such, this collection of glycans intrinsically allows for comparative analysis of carbohydrate structural determinants that are important for recognition and binding, and it becomes important to look at what structures are *bound* as well as related structures that are *unbound*. For example, examination of the sialic acid recognition of influenza A virus (IAV) hemagglutinin has been completed using the CFG array (Bradley et al., 2011a; Gulati et al., 2013, 2014; Byrd-Leotis et al., 2014), and those interactions are exclusively limited to the Neu5Ac form as seen in multiple studies using many IAV strains, with no binding to Neu5Gc (**Figure 1**), as visualized with the GLycan Array Dashboard (GLAD) analytics program (Mehta and Cummings, 2019). Interestingly, as swine isolates of IAV were studied on the CFG array, the lack of Neu5Gc binding was maintained, indicating that while such glycans are able to be synthesized within the swine host, they are still unable to be recognized by influenza HA, highlighting the specificity of the receptor binding pocket for the N-acetyl over the N-glycolyl moiety (Bradley et al., 2011a). In broader strokes, the comparative nature of the CFG array has been utilized extensively as a diagnostic indicator of pandemic potential for influenza A viruses as binding preference to terminal sialic acid linkage conformations that are indicative of avian (α2,3-Sia) or human (α2,6-Sia) adaptation are easily visualized (Gulati et al., 2013, 2014; Byrd-Leotis et al., 2014). While the synthetic glycan arrays are powerful tools to elucidate the possible recognition by the pathogen or host protein under examination, the biological relevance of such interactions cannot be known. The question is 2-fold: are the glycans that are synthesized in the laboratory representative of those in the host and if so, are they present in enough abundance and localized appropriately in order to be utilized as receptors? In order to study these questions, a different process for generating the glycan microarrays was developed.

Glycans From Natural Sources

Natural glycan microarrays are so designated because the glycans have been directly isolated from host tissues, functionalized, typically partly purified, and then printed on glass slides to create a microarray (**Figure 2**; Song et al., 2011a). These arrays are direct representations of the glycan profiles, including both structural content and relative abundance, of the target host tissue and therefore provide relevant biological information about the binding interactions. These arrays are often presented as shotgun arrays, meaning that the glycans are not sequenced prior to printing and as such, the investigators are reliant on interactions with GBPs to prioritize the glycan characterization. Multiple strategies for glycan release from the tissue have been outlined including enzymatic release (Heimburg-Molinaro et al., 2011; Song et al., 2014) and chemical release using sodium hypochlorite (NaOCl or bleach) (Song et al., 2016). Once the glycans have been released, the functionalization process varies widely with respect to the linkers used, the substrate for printing, and the methods of analysis. This strategy has generated great excitement across many fields of study to look at the natural receptors and binding partners for many biologically relevant questions, including the interactions of influenza viruses with the native host receptors. Because different sialic acids can be detected by several methods, including lectin binding, the natural arrays can begin to be characterized using reagents of known specificity to help categorize and map out some of the structural features present in the tissue/cells before any detailed structural information is obtained. A natural shotgun glycan array comprised of glycans from swine lungs reveals the presence of sialylated glycans within the tissue that are potential receptors for various strains of IAV (Byrd-Leotis et al., 2014). This work showed a practical, biologically-relevant use of the "shotgun glycomics" method with pig lung tissue and allowed for studies of the natural receptors for influenza. The presence of sialylated glycans was able to be discerned clearly with the use of chromatographic separation, mass spectrometry, and the binding of plant lectins with known specificity, before looking at virus binding. Because the binding specificities of viruses chosen for study had been established on the defined CFG array, predictions

TABLE 1 | List of sialylated glycans present on the CFG glycan microarray, representing about 20% of the total number of glycans present.

Sialylated glycans on CFG array	
Neu5Aca-Sp8	Neu5Aca2-6(Galb1-3)GlcNAcb1-4Galb1-4Glcb-Sp10
Neu5Aca-Sp11	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3Manb1-4GlcNAcb1-4GlcNAc-Sp12
Neu5Acb-Sp8	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12
Neu5Aca2-3(6S)Galb1-4GlcNAcb-Sp8	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12
Neu5Aca2-3Galb-Sp8	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(GlcNAcb1-4)(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21
Neu5Aca2-3Galb1-3(6S)GalNAca-Sp8	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Mana1-3)Manb1-4GlcNAcb1-4GlcNAc-Sp12
Neu5Aca2-3Galb1-3(6S)GlcNAc-Sp8	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Man-a1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21
Neu5Aca2-3Galb1-3(Fuca1-4)GlcNAcb-Sp8	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp24
Neu5Aca2-3Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-3(Fuca1-4)GlcNAcb-Sp0	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12
Neu5Aca2-3Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp24
Neu5Aca2-3Galb1-3GalNAca-Sp14	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6Manb1-4GlcNAcb1-4GlcNAc-Sp12
Neu5Aca2-3Galb1-3GalNAca-Sp8	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-3GlcNAcb-Sp0
Neu5Aca2-3Galb1-3GalNAcb1-3Gala1-4Galb1-4Glcb-Sp0	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0
Neu5Aca2-3Galb1-3GalNAcb1-4(Neu5Aca2-3)Galb1-4Glcb-Sp0	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0
Neu5Aca2-3Galb1-3GalNAcb1-4Galb1-4Glcb-Sp0	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12
Neu5Aca2-3Galb1-3GlcNAcb-Sp0	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0
Neu5Aca2-3Galb1-3GlcNAcb1-2Mana-Sp0	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12
Neu5Aca2-3Galb1-3GlcNAcb1-2Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-3GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAc-Sp21	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3GalNAca-Sp14
Neu5Aca2-3Galb1-3GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-3GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAc-Sp19	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14
Neu5Aca2-3Galb1-3GlcNAcb1-3Galb1-3GlcNAcb-Sp0	Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Neu5Aca2-6Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3)GalNAca-Sp14
Neu5Aca2-3Galb1-3GlcNAcb1-3Galb1-4GlcNAcb-Sp0	Neu5Aca2-6Galb1-4GlcNAcb1-3GalNAc-Sp14
Neu5Aca2-3Galb1-3GlcNAcb1-3GalNAca-Sp14	Neu5Aca2-6Galb1-4GlcNAcb1-4Mana1-6(GlcNAcb1-4)(Neu5Aca2-6Galb1-4GlcNAcb1-4(Neu5Aca2-6Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21
Neu5Aca2-3Galb1-3GlcNAcb1-6GalNAca-Sp14	Neu5Aca2-6Galb1-4GlcNAcb1-6(Fuca1-2Galb1-3GlcNAcb1-3)Galb1-4Glc-Sp21
Neu5Aca2-3Galb1-4(6S)GlcNAcb-Sp8	Neu5Aca2-6Galb1-4GlcNAcb1-6(Fuca1-2Galb1-4(Fuca1-3)GlcNAcb1-3)Galb1-4Glc-Sp21
Neu5Aca2-3Galb1-4(Fuca1-3)(6S)GlcNAcb-Sp8	Neu5Aca2-6Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14
Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	Neu5Aca2-6Galb1-4GlcNAcb1-6(Galb1-3GlcNAcb1-3)Galb1-4Glcb-Sp21
Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb-Sp8	Neu5Aca2-6Galb1-4GlcNAcb1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21
Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-2Mana-Sp0	Neu5Aca2-6Galb1-4GlcNAcb1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-6Galb1-4GlcNAcb1-4(Neu5Aca2-6Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21
Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb-Sp8	Neu5Aca2-6Galb1-4GlcNAcb1-6GalNAca-Sp14

(Continued)

TABLE 1 | Continued

Sialylated glycans on CFG array	
Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	Neu5Aca2-6GalNAca-Sp8
Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4GlcNAcb-Sp8	Neu5Aca2-6GalNAcb1-4(6S)GlcNAcb-Sp8
Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3GalNAca-Sp14	Neu5Aca2-6GalNAcb1-4GlcNAcb-Sp0
Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-6(Galb1-3)GalNAca-Sp14	Neu5Aca2-6GlcNAcb1-4GlcNAc-Sp21
Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-6(Neu5Aca2-3Galb1-3)GalNAc-Sp14	Neu5Aca2-6GlcNAcb1-4GlcNAcb1-4GlcNAc-Sp21
Neu5Aca2-3Galb1-4(Neu5Aca2-3Galb1-3)GlcNAcb-Sp8	Galb1-4GlcNAcb1-6(Neu5Aca2-6Galb1-3GlcNAcb1-3)Galb1-4Glc-Sp21
Neu5Aca2-3Galb1-4Glcb-Sp0	Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12
Neu5Aca2-3Galb1-4Glcb-Sp8	Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAc-Sp12
Neu5Aca2-3Galb1-4GlcNAcb-Sp0	Neu5Acb2-6GalNAca-Sp8
Neu5Aca2-3Galb1-4GlcNAcb-Sp8	Neu5Acb2-6(Galb1-3)GalNAca-Sp8
Neu5Aca2-3Galb1-4GlcNAcb1-2Mana-Sp0	Neu5Acb2-6Galb1-4GlcNAcb-Sp8
Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	Neu5Aca2-8Neu5Aca-Sp8
Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp24	Neu5Aca2-8Neu5Acb-Sp17
Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	Neu5Aca2-8Neu5Aca2-8Neu5Aca-Sp8
Neu5Aca2-3Galb1-4GlcNAcb1-3Galb-Sp8	Neu5Aca2-8Neu5Aca2-8Neu5Acb-Sp8
Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-3GlcNAcb-Sp0	Neu5Aca2-8Neu5Aca2-3Galb1-4Glcb-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	Neu5Aca2-8Neu5Gca2-3Galb1-4GlcNAc-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0	Neu5Aca2-8Neu5Aca2-3Galb1-4GlcNAc-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	Neu5Aca2-8Neu5Aca2-8Neu5Aca2-3Galb1-4Glcb-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0	Neu5Aca2-8Neu5Aca2-3Galb1-3GalNAcb1-4(Neu5Aca2-3)Galb1-4Glc-Sp21
Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	Neu5Aca2-8Neu5Aca2-3Galb1-3GalNAcb1-4(Neu5Aca2-8Neu5Aca2-3)Galb1-4Glcb-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3GalNAca-Sp14	GalNAcb1-4(Neu5Aca2-8Neu5Aca2-3)Galb1-4Glcb-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14	GalNAcb1-4(Neu5Aca2-8Neu5Aca2-8Neu5Aca2-8Neu5Aca2-3)Galb1-4Glcb-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3)GalNAca-Sp14	GalNAcb1-4(Neu5Aca2-8Neu5Aca2-8Neu5Aca2-3)Galb1-4Glcb-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-3GalNAc-Sp14	Galb1-3GalNAcb1-4(Neu5Aca2-8Neu5Aca2-8Neu5Aca2-3)Galb1-4Glcb-Sp21
Neu5Aca2-3Galb1-4GlcNAcb1-4Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-4(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	Neu5Gca-Sp8
Neu5Aca2-3Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14	Neu5Gca2-3Galb1-3(Fuca1-4)GlcNAcb-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-3)GalNAca-Sp14	Neu5Gca2-3Galb1-3GlcNAcb-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	Neu5Gca2-3Galb1-4(Fuca1-3)GlcNAcb-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-4(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	Neu5Gca2-3Galb1-4GlcNAcb-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3)GalNAca-Sp14	Neu5Gca2-3Galb1-4Glcb-Sp0
Neu5Aca2-3Galb1-4GlcNAcb1-6GalNAca-Sp14	Neu5Gca2-6GalNAca-Sp0
Neu5Aca2-3GalNAca-Sp8	Neu5Gca2-6Galb1-4GlcNAcb-Sp0

(Continued)

TABLE 1 | Continued

Sialylated glycans on CFG array	
Neu5Aca2-3GalNAcb1-4GlcNAcb-Sp0	Neu5Gcb2-6Galb1-4GlcNAc-Sp8
GalNAcb1-4(Neu5Aca2-3)Galb1-4GlcNAcb-Sp0	Neu5Gca2-8Neu5Gca2-3Galb1-4GlcNAc-Sp0
GalNAcb1-4(Neu5Aca2-3)Galb1-4GlcNAcb-Sp8	Neu5Gca2-8Neu5Aca2-3Galb1-4GlcNAc-Sp0
GalNAcb1-4(Neu5Aca2-3)Galb1-4GlcNAcb1-3GalNAca-Sp14	Neu5Gca2-8Neu5Gca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAc-Sp0
GlcNAcb1-6(Neu5Aca2-3Galb1-3)GalNAca-Sp14	Neu5Gca2-8Neu5Gca2-6Galb1-4GlcNAc-Sp0
Fuca1-2Galb1-3GalNAcb1-4(Neu5Aca2-3)Galb1-4Glcb-Sp0	Neu5,9Ac$_2$a-Sp8
Fuca1-2Galb1-3GalNAcb1-4(Neu5Aca2-3)Galb1-4Glcb-Sp9	Neu5,9Ac2a2-6Galb1-4GlcNAcb-Sp8
GalNAcb1-4(Neu5Aca2-3)Galb1-4Glcb-Sp0	Neu5,9Ac2a2-3Galb1-4GlcNAcb-Sp0
Galb1-3GalNAcb1-4(Neu5Aca2-3)Galb1-4Glcb-Sp0	Neu5,9Ac2a2-3Galb1-3GlcNAcb-Sp0
Neu5Aca2-3Galb1-3GalNAcb1-4(Neu5Aca2-8Neu5Aca2-3)Galb1-4Glcb-Sp0	
Neu5Aca2-6(Neu5Aca2-3)GalNAca-Sp8	
Neu5Aca2-6(Neu5Aca2-3Galb1-3)GalNAca-Sp14	
Neu5Aca2-6(Neu5Aca2-3Galb1-3)GalNAca-Sp8	**Linker structures**
Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	Sp0 : -CH2CH2NH2
Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	Sp8 : -CH2CH2CH2NH2
Galb1-4(Fuca1-3)GlcNAcb1-6(Neu5Aca2-6(Neu5Aca2-3Galb1-3)GlcNAcb1-3)Galb1-4Glc-Sp21	Sp9 : -CH2CH2CH2CH2CH2NH2
Neu5Aca2-6Galb1-4 GlcNAcb1-6(Neu5Aca2-6Galb1-4GlcNAcb1-3)GalNAca-Sp14	Sp10 : -NHCOCH2NH
Neu5Aca2-6Galb-Sp8	Sp11 : -OCH2C6H4-p-NHCOCH2NH
Neu5Aca2-6Galb1-4(6S)GlcNAcb-Sp8	Sp12 : Asparagine
Neu5Aca2-6Galb1-4Glcb-Sp0	Sp14 : Threonine
Neu5Aca2-6Galb1-4Glcb-Sp8	Sp17 : -OCH2C6H4NH2
Neu5Aca2-6Galb1-4GlcNAcb-Sp0	Sp19 : EN or NK
Neu5Aca2-6Galb1-4GlcNAcb-Sp8	Sp21 : -N(CH3)-O-(CH2)2-NH2
Neu5Aca2-6Galb1-4GlcNAcb1-2Man-Sp0	Sp24 : KVANKT
Neu5Aca2-6(Galb1-3)GalNAca-Sp14	
Neu5Aca2-6(Galb1-3)GalNAca-Sp8	

See the CFG website for more information. Spacer (linker) structures are provided in the table; a- or b- in the structure and prior to the Sp designation indicates the alpha or beta linkage; (6S) indicates sulfate groups and linkage. Yellow highlight indicates the structures that specifically contain Gc sialic acid as opposed to Ac or a derivative.

regarding the types of glycans on the natural array that would be recognized by IAV could be made. The predictions held true for many structures which were present on both arrays, but IAV binding also revealed interactions with new structures that were not present on the defined array, for example triantennary α2,3 and α2,6 Sia-terminating, core fucosylated N-glycans. As such, the natural shotgun glycan microarray of the pig lung exposed biologically relevant receptors for influenza A viruses that would not have been discovered by relying on the synthetic arrays alone.

A more recent study used a human lung shotgun glycan microarray, derived from total human lung glycoproteins, which would not be expected to contain Neu5Gc. Indeed, this was observed, as this glycan microarray helped to identify both sialylated (NeuAc), and phosphorylated oligomannose-type N-glycans recognized by various strains of influenza virus (Byrd-Leotis et al., 2019). The work complemented the development of a synthetic N-glycan microarray comprised of Asn-linked oligosaccharides terminated in various branching structures and with either α2,3- or α2,6-sialylation (Gao et al., 2019). Many recent influenza virus isolates showed preferential binding to the phosphorylated oligomannose-type N-glycans compared to sialylated glycans (Byrd-Leotis et al., 2019).

Specialized Sialo-Arrays and Tools

Many groups have generated specialized arrays for the study of specific glycan interactions. Arrays of microbial polysaccharides, which contain sialic acids as well as various derivatives such as 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN), have been generated to study many types of host-pathogen interactions, for example the examination of human serum antibodies and innate and adaptive immune proteins (Wang et al., 2002). Specific N-glycan microarrays have been generated with and without terminal sialic acid, and these arrays can be tested with GBPs to look for interactions with sialylated and non-sialylated structures, but in addition can be modified by enzymatic or chemical methods to create new sialylated epitopes with Neu5Ac, Neu5Gc, and other modified sialic acids for binding studies (Hamilton et al., 2017). An array of sialic acid

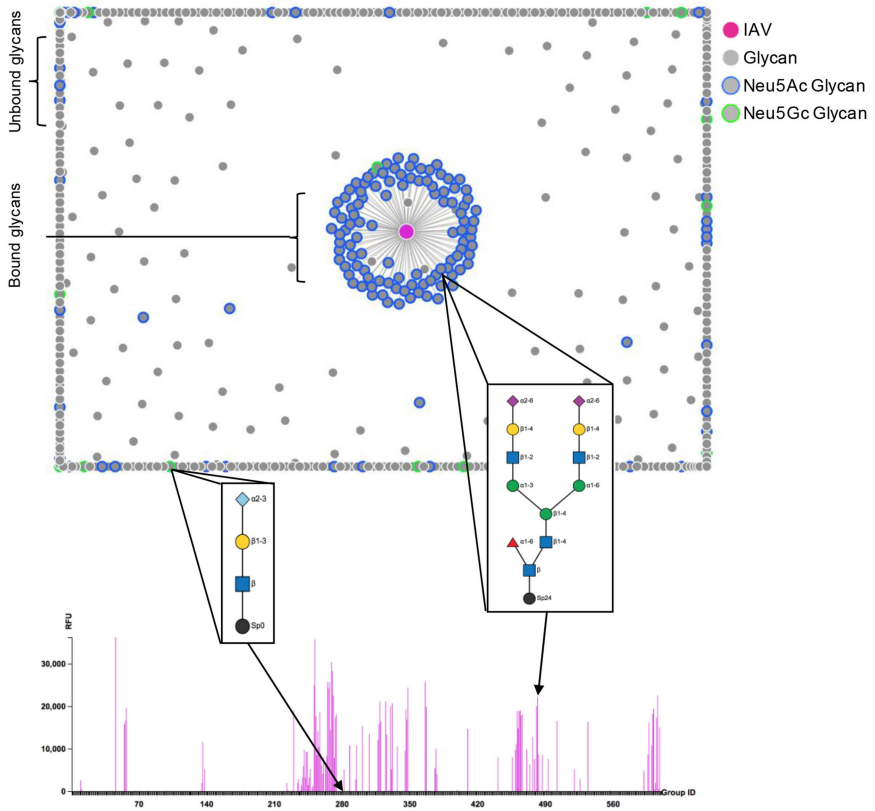

FIGURE 1 | Influenza A virus binding to CFG array. The strength of IAV binding to each glycan on the CFG array is visualized by the force plot, where bound glycans are linked to the IAV spot (magenta) in the middle and unbound glycans are pushed to the periphery. Neu5Ac and Neu5Gc sialylated glycans are highlighted in blue and green, respectively. The structures corresponding to one example each of Neu5Ac and Neu5Gc terminating glycans are displayed and the binding to each structure is highlighted on the bar chart comparing RFU. Glycan cartoon key: light blue diamond- Neu5Gc, purple diamond- Neu5Ac, yellow circle- Gal, blue square- GlcNAc, green circle- Man, red triangle- Fuc, black circle- linker. Figure produced using the GLycan Array Dashboard (GLAD) program, developed by the Cummings group (Mehta and Cummings, 2019), website- https://glycotoolkit.com/Tools/GLAD/.

derivatives, not found on the CFG array or other arrays, was created using a novel method to examine binding interactions with these more unusual sialylated compounds (Bradley et al., 2011b; Song et al., 2011b). The array was informative for lectin binding, as well as influenza and parainfluenza binding studies. While most influenza strains maintained a lack of binding to NeuGc derivatives, a new binding partner was seen- a lactoyl Neu5Ac derivative, and some of the parainfluenza viruses showed binding to Neu5Gc derivatives containing O-methyl groups. This type of derivative array expanded the diversity of sialic acid compounds that could be analyzed and broadened the knowledge on what types of glycans, in terms of charge, size, and other features, were capable of being bound by well-studied lectins and viruses. Glycoproteins have also served as useful materials to be printed on microarrays (Patwa et al., 2010). While they are inherently heterogeneous compared to defined glycans, they can provide interesting binding data. Some glycoproteins, such as fetuin, have well characterized glycans and so the binding to the glycoprotein can be informative, especially if coupled with treatments such as neuraminidase/sialidase treatments to remove terminal sialic acids.

As mentioned above, many novel sialidases generated by Chen's laboratory were utilized to create sialylated glycan microarrays with interesting modifications to sialic acids including Neu5Gc and 9-O-acetyl derivatives (Yu et al., 2005, 2006a,b), and these arrays were utilized to show the presence of antibodies in patients with carcinomas to a specific glycan, Neu5Gcα2–6GalNAcα1-*O*-Ser/Thr (GcSTn) (Padler-Karavani et al., 2011). Similar arrays were also used to show the novel finding of elevated anti-Gc antibodies in patients with Kawasaki Disease (Padler-Karavani et al., 2013), further demonstrating the usefulness of the array platform. A large-scale study using a sialoglycan microarray generated by the same research groups showed an association of multiple antibodies to Neu5Gc with colorectal cancer risk (Samraj et al., 2018). As described, both the synthetic and natural glycan microarrays have been used to examine a variety of interactions, including those mediated by viral and bacterial proteins, host antibodies, and serum.

The Neoglycolipid (NGL)-based microarray system uses defined glycans, synthetically or naturally obtained, linked to lipids which are then bound to a nitro-cellulose slide (Palma

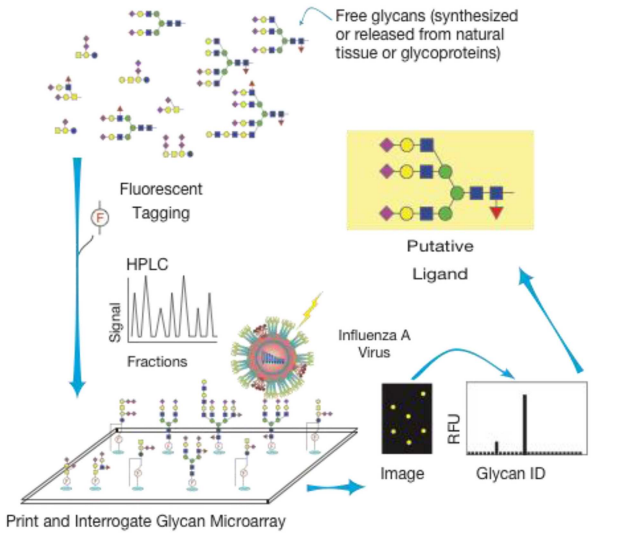

FIGURE 2 | General method for creation and use of glycan microarrays from natural materials.

et al., 2014). This platform has been useful in many instances, and was used by Feizi's laboratory to demonstrate that an N-glycolyl GM1-based glycan is a receptor for simian virus 40 (Campanero-Rhodes et al., 2007). They have incorporated various sialic acid-containing glycans into their NGL array platforms, including NeuGc and polysialic acid, and has also made discoveries on binding of other types of samples, such as human adenovirus 52 (Lenman et al., 2018). In addition, N-glycolyl sialic acid was discovered as a binding partner for human polyomavirus 9 viral proteins, further demonstrating the utility of these array formats (Khan et al., 2014).

Bifunctional linkers useful for both the detection of HPLC-separated glycan species and direct printing of glycans to NHS slides have been developed (Song et al., 2009, 2015; Yamada et al., 2013), such as AEAB and Fmoc derivatives, which allow for easy purification and quantification, in addition to the fluorescent properties and printing capabilities. Surface plasmon resonance (SPR) can be used in place of fluorescence as an indicator of binding interactions (de Boer et al., 2008). SPR studies coupled with glycan array studies have subsequently been used to look at sialic acid binding, specifically comparing the ability of proteins to bind to NeuAc vs. NeuGc, and making discoveries on host-pathogen interactions (Atack et al., 2018).

Limitations of Microarray Approaches

Microarrays have been an incredible tool for investigating binding interactions on a solid surface between the glycans covalently linked to slides and soluble GBPs. However, there are some limitations to the technique. The most straightforward issue is in the number of glycans that are available for addition to the microarrays. There are predicted to be thousands of glycans in the human glycan repertoire (Cummings, 2009; Cummings and Pierce, 2014), however only a fraction of these are accessible as known glycan structures for incorporation into arrays. There is also a restriction in the enzyme availability- there are not enzymes available to generate every known glycan linkage that has been identified. Additionally, some studies indicate that solution-based experiments are advantageous over solid phase. In order to increase accessibility and address the nature of in solution vs. fixed surface interactions, many groups are investigating the generation of glycan arrays on alternative substrates, such as beads, that will allow for flow cytometric analysis (Purohit et al., 2018). Similarly, cell-based arrays allow for a biologically relevant presentation of glycans (Briard et al., 2018).

SIALIC ACID BINDING REAGENTS

There are a number of reagents that are used in the field for identifying sialic acids. These include using plant or animal lectins, which are naturally occurring glycan-binding proteins, as well as anti-glycan antibodies. On the glycan microarrays, these reagents are detected fluorescently, either due to their direct fluorescence, or with a fluorescently labeled secondary reagent such as streptavidin.

Specifically, *Sambucus nigra* agglutinin (SNA), *Maackia amurensis* leukoagglutinin (MAL-I), and *Maackia amurensis* hemagglutinin (MAH or MAL-II) are all lectins that have been well-characterized in their binding motifs, favoring glycans that contain sialic acid. SNA is a bark lectin from the elderberry plant that has a high affinity toward structures containing terminal Neu5Acα2-6Galβ-. It has unique abilities to differentiate and bind favorably to α2-6-linked sialic acid (Sia)-containing ligands over α2-3-linked sialylated glycans (Cummings and Schnaar, 2017). Binding of SNA to the CFG glycan array shows a strong preference to the Ac version of sialic acid over the Gc, with only one of the Gc compounds being bound. MAL-I and MAL-II are both derived from the leguminous tree, *M. amurensis,* but have diverse binding profiles and affinities. MAL-I has consistently shown affinity toward terminal Neu5Acα2-3 residues that are linked to type-2 N-acetyllactosamine sequences, such as Siaα2-3Galβ1-4GlcNAcβ-Man-R. Studies have shown that this lectin does not bind isomers that contain sialic acid in a α2-6 linkage, with strong preference for α2-3 linkages. MAL-I has also shown binding to glycans that are sulfated as opposed to sialylated with the typical sequence, sulfo-3Galβ1-4GlcNAcβ-Man-R (Cummings and Schnaar, 2017). Analyzing data from MAL-I on the CFG array revealed high binding toward gangliosides that have Gc in their structure, either in a α2-3 or α2-8 linkage, in addition to the Ac and negatively-charged sulfate binding. The binding is less influenced by the Gc and Ac versions of sialic acid than SNA. MAL-II has distinct binding to sialylated core 1 O-glycan Siaα2-3Galβ1-3GalNAcα1-Ser/Thr. It does not exhibit binding to the Gc compounds present on the CFG array. The specificity of these lectins tested on other array platforms shows that it is not just the presence of Ac or Gc sialic acid that effects binding, but that the underlying structure is important, and these specificities are described in more detail in the respective publications (Padler-Karavani et al., 2011, 2012; Song et al., 2011b; Wang et al., 2014).

Commercially available antibodies that are specific in recognizing sialic acid are difficult to find, but the companies Biolegend and Lectenz Bio (www.Lectenz.com) have reagents

designated for this purpose. These reagents provide the field with more screening tools for biological samples. Lectenz Bio has a reagent that specifically targets α2-3 linked sialo-glycans over α2-6 and α2-8 linked sialo-glycans, which is similar binding specificity to MAL-I. Another anti-glycan reagent produced by Lectenz Bio aims to broadly identify glycans containing sialic acid in general, independent of the linkage. It remains to be seen whether these reagents can discriminate Ac and Gc. The anti-Neu5Gc antibody from Biolegend is particularly important in studies looking at the effects of the intake and incorporation of Neu5Gc in humans, which has been associated with inflammation and worsening of some diseases (Samraj et al., 2017). The anti-Gc antibodies appear to be specific for Gc compounds and not Ac compounds, and these antibodies are the subject of another review in this series (Dhar et al., 2019).

COMPARATIVE ANALYSIS OF GLYCAN MICROARRAYS AND DATA OUTPUT

All aspects of glycan microarray technology have advanced significantly from chemical and enzymatic generation of the glycans, to novel release methods, to the development of more efficient functional linkers and immobilization strategies (Gagarinov et al., 2017). As the field continues to develop, we are able to further refine the assays and find new uses for the existing glycan microarrays, as well as modify the existing structures on both defined and natural arrays to create new epitopes for binding studies. The MAGS approach (Smith and Cummings, 2013) has been used in conjunction with MS data to sequence unknown glycans, however the same process can be viewed from the perspective of characterizing relevant enzymes and lectins, such as defining the acute specificity of bacterial neuraminidases or the binding nuances of common lectins. Additionally, as more data is generated, a comprehensive comparative approach allows for a link to be established between existing glycoproteomics databases and glycan microarray data. The lectin-glycan interaction (LGI) network enables the prediction of host receptor proteins for pathogenic adhesins (Ielasi et al., 2016). The incredible volume of data generated from the various synthetic and natural glycan microarrays will be invaluable as more is discovered about the GBPs and predictive analysis is developed.

One head-to-head comparison of two similar but novel sialic acid derivative arrays was performed, using the same well-characterized lectins and proteins, and a cross-analysis of the slide images was performed to assess variability (Padler-Karavani et al., 2012). The arrays contained many glycans that were structurally identified, and others with subtle differences in comparison. They were also printed on 2 different slide surfaces, which was noted as a major contributing factor to the differences in binding profiles. The data demonstrates that arrays with the same or similar types of compounds can be complementary to one another. Nevertheless, they should also be compared between laboratories with caution, since there are many variables that contribute to data acquisition, including slide surface, printing method and concentration, buffer conditions, and analysis methods (Padler-Karavani et al., 2012), although we and other groups see very consistent results in terms of reproducibility within the same print batches across many types of glycans and linkers. This and other studies have shown that the properties and lengths of linkers used, the immobilization strategy and slide matrix chemistry, and density of immobilized glycans or glycoproteins can all affect recognition and therefore need to be considered in the data analysis and interpretation steps, and variations in these components could lead to differences in binding across different array platforms (Song et al., 2009; Padler-Karavani et al., 2012; Wang et al., 2014; Gildersleeve and Wright, 2016; Gao et al., 2019; Temme et al., 2019).

The MIRAGE efforts were initiated to aid in the cross-comparison of arrays. MIRAGE, or the Minimum Information Required for A Glycomics Experiment, has been setting the standards in the field for performing and reporting on the array methods and data analysis (Kolarich et al., 2013; York et al., 2014; Struwe et al., 2016; Liu et al., 2017; Campbell et al., 2019). These efforts will continue to benefit the collection of consistent array data across different types of arrays and from different research groups.

Another effort that is paving the way for cross-analyses and centralization of array data is the GlyGen program, https://www.glygen.org. This NIH-funded program is a resource for the glycoscience fields, as a data integration and dissemination tool. Data is retrieved from multiple international sources related to glycan microarrays and other Glycomics data as well as information on the proteins that are tested, and through bioinformatics approaches the data is integrated into a common portal to allow detailed searches and exploration of data. These cross-comparison and integration efforts will allow for validation of data obtained from the myriad of sialic acid-containing glycan microarrays that are currently present in the field.

CONCLUDING REMARKS

The platform of glycan microarrays, and specifically arrays containing sialic acid derivatives such as Neu5Ac and Neu5Gc, have greatly accelerated studies of GBPs and their glycan interactors. These efforts will continue to expand and diversify as the significance of glycan recognition for glycoscience in human immunity, cancer therapy, and host-pathogen interactions is better understood and appreciated, and as new tools are introduced to the research community.

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N-Glycolylneuraminic Acid (Neu5Gc) Null Large Animals by Targeting the CMP-Neu5Gc Hydroxylase (CMAH)

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The two major sialic acids described in mammalian cells are the N-glycolylneuraminic acid (Neu5Gc) and the N-acetylneuraminic acid (Neu5Ac). Neu5Gc synthesis starts from the N-acetylneuraminic acid (Neu5Ac) precursor modified by an hydroxylic group addition catalyzed by CMP-Neu5Ac hydroxylase enzyme (CMAH). In humans, CMAH was inactivated by a 92 bp deletion occurred 2–3 million years ago. Few other mammals do not synthetize Neu5Gc, however livestock species used for food production and as a source of biological materials for medical applications carry Neu5Gc. Trace amounts of Neu5Gc are up taken through the diet and incorporated into various tissues including epithelia and endothelia cells. Humans carry "natural," diet-induced Anti-Neu5Gc antibodies and when undertaking medical treatments or receiving transplants or devices that contain animal derived products they can cause immunological reaction affecting pharmacology, immune tolerance, and severe side effect like serum sickness disease (SSD). Neu5Gc null mice have been the main experimental model to study such phenotype. With the recent advances in genome editing, pigs and cattle KO for Neu5Gc have been generated always in association with the αGal KO. These large animals are normal and fertile and provide additional experimental models to study such mutation. Moreover, they will be the base for the development of new therapeutic applications like polyclonal IgG immunotherapy, Bioprosthetic Heart Valves, cells and tissues replacement.

Keywords: Neu5Gc, CMAH, αGal, GGTA1, knock out, cattle, pig

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INTRODUCTION

Sialic acids are monosaccharide expressed on the cell surface. They are incorporated as terminal residues in different types of glycoproteins and glycolipids. They are responsible for cell-to-cell and cell-to-microenvironmental interactions. The two major sialic acids described in mammalian cells are the N-glycolylneuraminic acid (Neu5Gc) and the N-acetylneuraminic acid (Neu5Ac). Neu5Gc synthesis starts from the N-acetylneuraminic acid (Neu5Ac) precursor modified by an hydroxylic group addition catalyzed by CMP-Neu5Ac hydroxylase enzyme (1). In non-human mammals, the CMP-Neu5Ac hydroxylase enzyme is coded by the CMAH gene, that was inactivated in humans by a 92 bp deletion that occurred 2–3 millions years ago (2, 3). Only recently have new studies demonstrated that CMAH inactivation occurred independently in the new world monkeys (4) and in other mammals (5), suggesting that the Neu5Gc loss and production of protective Anti-Neu5Gc antibodies could be explained in different mammals as an evolutionary resistance mechanism to different pathogens (6).

The CMAH gene was firstly knocked out in mice (7) generating several "human-like" phenotypes such as the induction of Anti-Neu5Gc antibodies with a possible deleterious interaction with endothelia and epithelia after nutritional incorporation of Neu5Gc, a trend for increased inflammation and immune responses, enhanced immune clearance of recombinant Neu5Gc therapeutics (8), delayed skin wound healing, age-related hearing loss, sexual selection through Neu5Gc antigenicity (9), altered susceptibility to muscular dystrophy (1, 10). Indeed, although humans cannot synthetize the CMP-Neu5Ac hydroxylase enzyme, traces of Neu5Gc are integrated into the membranes of human epithelial and endothelial cells (EC) and more efficiently in malignant cells via food intake. The red meats (beef, pork, and lamb, particularly in processed forms) and dairy products have high contents of Neu5Gc residues, while they are absent in poultry and fish. However, a simple Neu5Gc rich diet exposure, cannot elicit Anti-Neu5Gc antibodies in $CMAH^{-/-}$ mice. In fact, specific Anti-Neu5Gc antibodies were obtained only following strongly immunogenic agents (such as xenogeneic cells) or when a human-specific commensal/pathogen (as the non-typable Haemophilus influenza = NTHi) expressing some adsorbed Neu5Gc residues were used (11). The low antigenic properties of purified Neu5Gc residues was confirmed by another group (12) and, more recently, by Frei et al. immunizing the Neu5Gc-null mice with non-microbial Neu5Gc (13).

Although the possibility that diet induced Anti-Neu5Gc antibodies could have pathological effects in vascular diseases or oncogenesis is still debated in humans, there is evidence for the role of Anti-Neu5Gc in rejection of animal derived tissues [see Salama et al. for review (14)]. Anti-Neu5Gc are elicited by engineered pig skin (15, 16). Pancreatic islets are rejected by $CMAH^{-/-}$ null recipients mice after allotransplantation (12). However, in contrast to Anti-Gal, the presence of Anti-Neu5G antibodies does not seem to trigger hyperacute vascular rejection of Neu5Gc positive chimpanzee kidney in humans [reviewed in Salama et al. (14)] or in a islets transplantation model in Neu5Gc KO mice recipients (12). (See also the detailed review of the role of the elicited Anti-Neu5Gc antibodies recipients of organ and tissue xenotransplantation by Bach et al. in this journal issue). Anti-Neu5Gc antibodies also contribute to modify the pharmacology of therapeutic animal polyclonal IgG (14). In this last case, the rise of high titers of elicited Anti-Neu5Gc antibodies likely contributes to the serum sickness diseases occurring in almost all non-immunosuppressed patients (17, 18).

Developing large animals lacking the major xeno-antigens has thus become a key issue to provide animal vascularized or engineered xenogeneic tissues or glycosylated molecules lacking major xeno-antigens such as αGal, Neu5Gc, or β4GalNT2 in order to decrease the acute or delayed xenograft rejection mediated by the corresponding antibodies. Furthermore, the absence of Neu5Gc will alleviate the possible deleterious effect of elicited Anti-Neu5Gc antibodies on the xenograft recipients own endothelial cells displaying traces of dietary derived Neu5Gc. Finally, it is possible that some patients with red meat intestinal allergy develop IgE Anti-Neu5Gc antibodies (as for Anti-Gal IgE), suggesting by analogy that meat and milk also lacking Neu5Gc, may alleviate such symptoms. In this article, we review the methodological advances that allowed the engineering of pigs and bovine lacking the Neu5Gc alone or in association to other xeno antigens and their potential future value in human pathology and health.

METHODOLOGICAL ASPECTS OF GENE CMAH (CYTIDINE MONOPHOSPHATE-N-ACETYLNEURAMINIC ACID HYDROXYLASE) INACTIVATION IN LARGE ANIMALS

The last 10 years have seen the advent and development of programmable nucleases for precise editing of the genome (19–22). What was used in the past to achieve genetic modification essentially was a by-product of the cell DNA repair mechanisms (NHEJ, non-homologous end-joining or HDR, homology directed repair), taking advantage of the double strand DNA breaks occurring spontaneously at a very low pace throughout the genome. Now the frequency of DNA breaks is enhanced by a few logs times by the precise cutting ability of the programmable nucleases at selected target sequences. Amongst the programmable nucleases used today for genome editing (**Table 1**), CRISPR/Cas9 is the most widely used because of its ease to use, flexibility (23), and low cost. The full exploitation of these technologies requires accurate DNA sequencing data as well as the software tools necessary for nuclease design, target site selection, and experimental validation (24–26) and to avoid undesired side effects in other genomic loci. Moreover, compared to the previous techniques of gene targeting, nuclease-based approach does not require the insertion of foreign DNA in the form of selectable markers. These types of nucleases have all been used to successfully edit the genome in a variety of organisms including livestock species both for agricultural (27, 28) and biomedical applications (29, 30), to mention only a few. In the biomedical arena, one species of long standing interest for genetic modification has been the pig for xenotransplantation research, usually targeting one specific locus (31), or two (32). More recently, CRISPR/Cas9 has been implemented to a degree of efficiency to obtain the multiple simultaneous mutations of 3 xenoantigens in the pig (33, 34), including the CMAH gene responsible for the synthesis of Neu5Gc. Even more impressive has been the work reported by Niu et al. (35) with the simultaneous inactivation of all copies of PERVs in the porcine genome. The KO of the enzyme CMAH (cytidine monophosphate-N-acetylneuraminic acid hydroxylase) responsible for the Neu5Gc antigen in pigs has been achieved by several groups, always in association with the simultaneous targeting of the α(1,3) galactosyltransferase (encoded by the GGTA1 gene), using Zn-finger nucleases (32, 36), with TALENs (37), with CRISPR/Cas9 (34), and more recently by the KO of β4GalNT2 (β1,4-N-acetylgalactosaminyltransferase) gene, done simultaneously with the KO of GGTA1 and CMAH (33), again with CRISPR/Cas9. However, there is no public information on breeding successive generation of these pigs, nor is it available

TABLE 1 | Comparison of different programmable nuclease platforms used in livestock genome editing [adapted from Cox et al. (22) with permission from the Publisher].

	Zinc finger nuclease	TALEN	Cas9
Recognition site	Typically 9–18 bp per ZFN monomer, 18–36 bp per ZFN pair	Typically 14–20 bp per TALEN monomer, 28–40 bp per TALEN pair	22 bp [20-bp guide sequence + 2-bp protospacer adjacent motif (PAM) for *Streptococcus pyogenes* Cas9]; up to 44 bp for double nicking
Specificity	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated
Targeting constraints	Difficult to target non-G-rich sequences	Five targeted base must be a T for each TALEN monomer	Targeted sequence must precede a PAM
Ease of engineering	Difficult; may require substantial protein engineering	Moderate; requires complex molecular cloning methods	Easily re-targeted using standard cloning procedures and oligo synthesis
Immunogenicity	Likely low, as zinc fingers are based on human protein scaffold; FokI is derived from bacteria and may be immunogenic	Unknown; protein derived from *Xanthamonas* sp.	Unknown; protein derived from various bacterial species
Ease of *ex vivo* delivery	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction
Ease of *in vivo* delivery	Relatively easy as small size of ZFN expression cassettes allows use in a variety of viral vectors	Difficult due to the large size of each TALEN and repetitive nature of DNA encoding TALENs, leading to unwanted recombination events when packaged into lentiviral vectors	Moderate: the commonly used Cas9 from *S. pyogenes* is large and may impose packaging problems for viral vectors such as AAV, but smaller orthologs exist
Ease of multiplexing	Low	Low	High

on pigs KO only for Neu5Gc. We have generated such animals by breeding αGal and Neu5GC KO male founders (DKO) to wild type sows. Since these mutations are transmitted in a Mendelian fashion, we were able to select in the F2 generation animals KO only for CMAH at the expected frequency (our unpublished observations). Neu5Gc null females were bred to DKO boars and reproduced normally, therefore although there has not been a systematic evaluation of significant numbers, there is no evidence that Neu5Gc KO pigs have major health or welfare issues, nor issues regarding growth rate or breeding ability. Since abnormal islets have been claimed to be associated to the $CMAH^{-/-}$ phenotypes (38), we further assessed the morphology and functionality of pancreatic islets from DKO pig compared to wild type counterparts (39). *In vitro* DKO pig islets exhibited normal insulin secretion after stimulation, there were also no islet histological abnormalities suggesting that the background of KO mice could have been responsible for such phenotype.

In our laboratories, we have recently generated the first cattle line knock out for both αGal and Neu5Gc using CRISPR/Cas9 technology and immunobeads selection (40). We have selected bovine fibroblasts carrying the bi-allelic inactivation of two enzymes including α(1,3) galactosyltransferase (encoded by the GGTA1 gene) and CMP-Neu5Gc hydroxylase (encoded by the CMAH gene) that are not functional in humans. Then, using somatic cell nuclear transfer (41) we generated live calves that do not express the two antigens (**Figure 1**). Because of the long generation interval in cattle compared to the pig, we have successfully edited both males and females founders. One male founder has reached puberty and semen was collected and cryopreserved for breeding purposes. Ejaculation parameters are normal and when the semen was used for *in vitro* fertilization, blastocyst stage embryos were obtained (our unpublished observations) at the same rate of WT bulls, demonstrating its fertility.

Neu5Gc-Null Large Animals in Applications and Diseases

The potential value of the Neu5Gc KO in biomedical or biotechnology applications is currently investigated in KO mice (42) or on *in vitro* models (43), given that large animal models KO for Neu5Gc have only recently become available. Clinical and experimental evidences demonstrated that when animal derived biologicals including cells, tissues or molecules such as Ig, are implanted or administered to patients, there is a strong Anti-Neu5Gc immune response, detectable years after the immune challenge such as with non-decellularized pig skin (16). High titers of Anti-Neu5Gc antibodies are also observed in patients receiving rabbit polyclonal IgG, all of them presenting a serum sickness disease (SSD) in the absence of immunosuppressants (IS) (18). The possibility that elicited Anti-Neu5Gc may induce vascular complication has been recently addressed in humans using experimental conditions closer to physiological ones with immune affinity purified diet derived and elicited Anti-Neu5Gc, and endothelial cells loaded with physiological amount of Neu5Gc (44). These data do not suggest that elicited (or diet derived) Anti-Neu5Gc induce an inflammation of endothelial cells but rather suggest that Anti-Neu5Gc may contribute to the homeostasis of ECs. These data are in agreement with the fact that all humans harbor substantial level of Anti-Neu5Gc which has not been eliminated by evolution. Why elicited Anti-Neu5Gc Abs do not result in a clear inflammatory profiling of the EC transcriptome remains unknown. Circulating Anti-Neu5Gc Abs show extremely large differential reactivities with multiple Neu5Gc-containing glycans in an array format. Such a huge epitope conformational diversity may result in a low

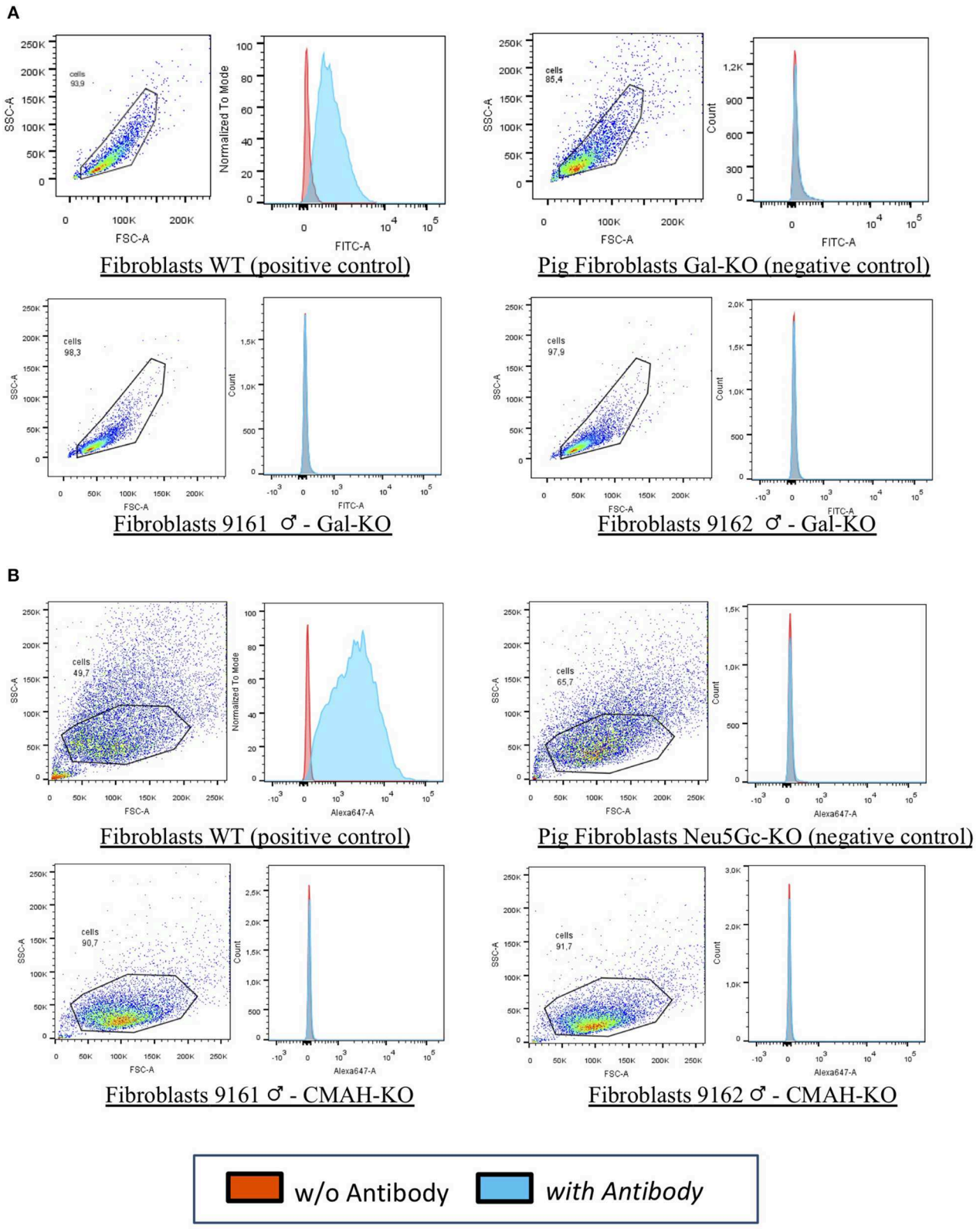

FIGURE 1 | FACS analyses for 9161 and 9162. Fibroblasts from wild type animal (WT) and from the edited calves 9161 and 9162 were analyzed by FACS. As negative controls pig DKO fibroblasts were used as no bovine material was available. The results demonstrated that the αGal **(A,B)** Neu5Gc antigens were absent from the cell surface of cloned calves, confirming the genotype analyses for the knocked-out genes (GGTA1 and CMAH). **(A)** Fibroblasts WT (positive control): wild

(Continued)

FIGURE 1 | type primary fibroblasts from the bovine line prior to genetic modification expressing the αGal and the Neu5Gc antigens. Pig Fibroblasts Gal-KO and Neu5Gc-KO (negative control): porcine primary fibroblasts NOT expressing the αGal and the Neu5Gc antigens. Fibroblasts 9161/9162 Gal-KO and Neu5Gc-KO: primary fibroblasts derived from cloned DKO calves [from Perota et al. (40) with permission of the publisher].

concentration of Abs for a given EC surface membrane target. Such a situation combined with the yet unknown mechanisms shaped by evolution to cope with the condition resulting from the concomitant presence of natural Anti-Neu5Gc Abs and their target on EC may ultimately result in signals that remain below the activation threshold of ECs. To some extent, Anti-Neu5Gc Abs may thus behave as other "natural antibodies" found in all normal human sera that react against a variety of autologous antigens. Beside the specific safety issue linked to severe SSD, occurrence of a serum sickness disease in patients treated by polyclonal IgGs of animal origin for severe infectious diseases or toxins related in non-immunosuppressed patients, it would also add its own symptomatology to the initial diseases for which the polyclonal antibodies were used. The availability of low immunogeneic, animal derived IgG for therapeutic purposes in non-IS patients is therefore a crucial need (45). In immunosuppressed patients, the Anti-Neu5Gc response resists more to immunosuppressive drugs than the Anti-Gal one does (17), it is however conceivable that Ig from Neu5Gc KO donor would substantially decrease the early toxicity of animal polyclonal IgG administered in the absence of IS such as in the case of Anti-toxins or Anti-infectious agents polyclonal preparations (46). Thus, given the important potential of polyclonal IgG therapeutic applications (47) and the high incidence of SSD mentioned in all studies in patients receiving the foreign Ig in the absence of immune suppression, the generation and availability of low immunogeneic animal derived Igs would be crucial. Approaches to produce human polyclonal antibodies in cattle and to decrease the protein backbone antigenicity included transchromosomic calves KO for the bovine immunoglobulin genes but carrying, on an artificial chromosome, the sequence of the human ones. However, in order to have an efficient immunoglobulin production in these animals, the authors had to "bovinize" the human genes by replacing the constant domain of the human genes (48, 49) with that of cattle creating chimeric immunoglobulins. However, because of the presence of the bovine backbone these antibodies still carry Neu5Gc and therefore will be highly immunogenic. The availability of cattle KO for Neu5Gc will solve this issue and may provide more safety and efficacy to polyclonal antisera immunotherapy, not only in cattle but also in other species used for these purposes like horse, pig, goat and sheep. For the same purpose and more specifically for bioprosthetic heart valves (BHV) manufacturing (seventy per cent of the BHV currently used in the clinic, are in fact manufactured with bovine pericardia), we have generated a cattle line knock out for both αGal and Neu5Gc (40). Although there are reports indicating that the current protocols for processing tissues for BHV manufacturing does not eliminate αGal (50) or Neu5Gc (51) there is yet no published report on the possible Anti-Neu5Gc antibodies response against pig or bovine Bioprosthetic Heart Valves (BHV), Anti-Neu5Gc antibodies may play a role in SVD (Structural Valve Degeneration), preventing the use of BHV in young patients (52, 53). BHV are routinely used in aortic valve replacements but, even if they are made using glutaraldehyde fixed porcine or bovine tissues (valves or pericardium), glutaraldehyde treatment is not sufficient to remove all the donors' glycans xenoantigens. Detection of αGal antigens (50) and of Neu5Gc residues (51), on BHV currently on the market, has been clearly demonstrated, suggesting by analogy a possible role for an immune based mechanism contributing to the SVD of BHV (54–57). To the best of our knowledge there is no study with sufficient statistic power demonstrating the association of BHV elicited anti-Neu5Gc and SVD. Of note the European sponsored FP7 Translink program is currently gathering the data obtained on a large multicentre cohort of BHV recipients where Anti-Neu5Gc levels and repertoire (as well as Anti-Gal) have been measured. Data from this study are expected for early 2020.

Neu5Gc-Null Large Animals in Food and Health

A largely unexplored avenue is the relevance of Neu5Gc intake through the diet of livestock derived products, both red meat and dairy products, especially for their high Neu5Gc content. As discussed above, diet is the source of a low level Neu5Gc and its accumulation in epithelial and endothelial cells where it could become the target of Anti-Neu5Gc antibodies. The level of such Anti-Neu5Gc antibodies is variable amongst individuals (44, 58–60) and it is not known if the presence of these "xeno auto antigens" is deleterious, as hypothesized (61). If Neu5Gc is incorporated into human tissue through the diet, at least in some individuals then the consumption of red meat or dairy products coming from KO animals should avoid the accumulation into tissues and reduce this risk factor. If this hypothesis will be further validated with additional evidence, then Neu5Gc KO animals could find their way into the food chain for some categories of individuals or infants at risk for specific diseases. Delayed allergic reaction following red meat consumption in some individuals, previously sensitized to αGal following a tick bite and exhibiting a stress-induced T helper-2 (Th2) shift favoring the production of IgE Anti-Gal, is an emergent problem. This allergic reaction, defined as read meat syndrome, has been shown to be related to a type 2 phenotype shift with the production of IgE Anti-Gal (62, 63), may also happen if IgE Anti-Neu5Gc are produced. For these reasons, molecules that are absent in humans but present in large animals that are used as a potential source of bio-products, have been knock out using genetic engineering techniques combined with Somatic Cell Nuclear Transfer (SCNT, animal cloning). αGal has been knock out in the pig by several research groups (64, 65) and this genetic background has become the basis on which to build

further genetic modifications for xenotransplantation research. Because of its absence in humans, and its presence in most mammals, Neu5Gc was the second target for genetic engineering for its high immunogenicity in humans as described above.

CONCLUSIONS

Contrary to the αGal antigens, that are immediately degraded, the Neu5Gc antigens from diet are integrated daily by the human cells in their carbohydrates, becoming a constant potential xeno-autoantigen in humans. The continuous exposure to Neu5Gc residues, present in the diet through products of animal origin or used as vital supports in various medical treatments, may theoretically result in a state of persistent inflammation tentatively called "xenosialitis." This condition has been proposed as an explanation of various diseases that characterize our species, despite the elimination of the Neu5Gc antigen and the development of Anti-Neu5Gc antibodies in humans having been an evolutionarily advantage in resisting to pathogens (66). Further studies are needed to assess the eventual susceptibility of Pigs and Cattle lacking Neu5Gc to human pathogens like for example Influenza A Virus (IAV). It is known for many years that the ferret is the best model for IAV infection studies and it has been demonstrated that the ferret lacks Neu5Gc (67). The availability of Neu5Gc KO livestock (cattle and pigs) that are our main source of animal protein can offer innovative solutions and opportunities in various fields. As it has been the case for the mouse, the first KO model available, the effect of such mutations on the pathophysiological state of animal models closer to humans can be studied in more details. A further possibility, offered by large animal KO models, is to use them as an "humanized" model in xenotransplantation research or diseases modeling. With such KO pigs and cattle it is now possible to source bioproducts for use in medical treatments from less immunogenic sources with great potential benefits (safety and efficacy) to the patients. Last but not least, if the dietary accumulation of Neu5Gc turns out to be a risk factor for some category of human patients, then there is a scope to breed such Neu5Gc-null livestock for food production as well.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

AP and CG reviewed the literature and wrote the paper.

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Conflict of Interest: AP and CG are employed by Avantea.

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Glycosylated Biotherapeutics: Immunological Effects of N-Glycolylneuraminic Acid

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The emerging field of biotherapeutics provides successful treatments for various diseases, yet immunogenicity and limited efficacy remain major concerns for many products. Glycosylation is a key factor determining the pharmacological properties of biotherapeutics, including their stability, solubility, bioavailability, pharmacokinetics, and immunogenicity. Hence, an increased attention is directed at optimizing the glycosylation properties of biotherapeutics. Currently, most biotherapeutics are produced in non-human mammalian cells in light of their ability to produce human-like glycosylation. However, most mammals produce the sialic acid *N*-glycolylneuraminic acid (Neu5Gc), while humans cannot due to a specific genetic defect. Humans consume Neu5Gc in their diet from mammalian derived foods (red meat and dairy) and produce polyclonal antibodies against diverse Neu5Gc-glycans. Moreover, Neu5Gc can metabolically incorporate into human cells and become presented on surface or secreted glycans, glycoproteins, and glycolipids. Several studies in mice suggested that the combination of Neu5Gc-containing epitopes and anti-Neu5Gc antibodies could contribute to exacerbation of chronic inflammation-mediated diseases (e.g., cancer, cardiovascular diseases, and autoimmunity). This could potentially become complicated with exposure to Neu5Gc-containing biotherapeutics, bio-devices or xenografts. Indeed, Neu5Gc can be found on various approved and marketed biotherapeutics. Here, we provide a perspective review on the possible consequences of Neu5Gc glycosylation of therapeutic protein drugs due to the limited published evidence of Neu5Gc glycosylation on marketed biotherapeutics and studies on their putative effects on immunogenicity, drug efficacy, and safety.

Keywords: antibody, biotherapeutics, glycosylation, sialic acid, *N*-glycolylneuraminic acid (Neu5Gc), immunology, anti-carbohydrate antibodies

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INTRODUCTION

Biotherapeutics are a rapidly increasing portion of the pharmaceutical market, with over a 100 new products approved and marketed in the U.S. and the European Union over the past decade (1). Among the commonly used biotherapeutics are antibodies, cytokines, enzymes, and hormones, originally purified from living organisms and characterized by their therapeutic potential, with limited evaluation of their potential immunological effects in recipient patients. Large-scale manufacturing of these therapeutic products involves expression of recombinant DNA in biological

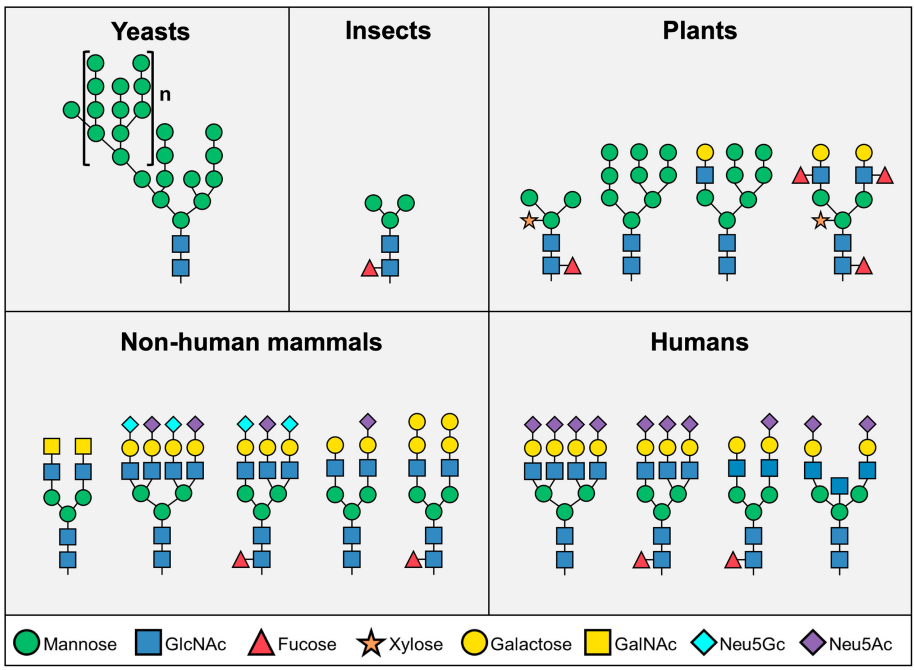

FIGURE 1 | Representative *N*-glycosylation pattern produced in different organisms. *N*-linked glycosylation process starts with biosynthesis of a common core structure of Man3GlcNAc2, but additional modifications varies significantly among species. Yeast cells typically produce high-mannose type glycans, while most insect *N*-glycans are composed of the core structure, and to a lower extent, additional mannose, fucose, and galactose. Plant cells produce more complex type glycans, often containing xylose. Mammalian cells mainly synthesize "human-like" complex type *N*-glycans, however human cells do not express the two common mammalian glycan antigens αGal and Neu5Gc (Man – mannose; GlcNAc – *N*-acetylglucosamine; GalNAc – *N*-acetylgalactosamine; Neu5Gc – *N*-glycolylneuraminic acid; Neu5Ac – *N*-Acetylneuraminic acid).

systems such as bacteria, yeast, insect and mammalian cells, as well as transgenic animals (1, 2). Being produced in living systems, therapeutic proteins often undergo post-translational modifications, most notably glycosylation.

Glycosylation is an important and ubiquitous modification, in which sugar chains (glycans) are covalently attached to proteins or lipids. Glycan biosynthesis is a template-independent process, which rely on a complex network of serially operating glycan-modifying enzymes (3, 4). The variety of possible monosaccharide compositions and modifications, linkage configurations and branching points gives rise to a tremendous diversity of glycan structures (Varki et al., 2015). Since this is not a template-driven process, proteins with identical amino acid sequences would typically differ in the degree of occupancy of their glycosylation sites (macro-heterogeneity), and would carry different glycans in a specific glycosylation site (micro-heterogeneity) (5). The glycosylation pattern of a cell changes through development and differentiation, under different environmental conditions, and during pathologies such as inflammation and malignancy, indicating the involvement of glycans in numerous processes in physiology and in disease (6).

Glycosylation of biotherapeutics has a substantial impact on their pharmacological properties and biological activity (7–10). Biotherapeutics glycosylation is largely determined by their production system (**Figure 1**). While non-mammalian cells (i.e., yeast, insect, or plant cells) are attractive due to their high yields, production of most biopharmaceutical products have shifted into mammalian expression platforms (i.e., hamster, human, or mouse cells) largely owing to consideration of their different glycosylation patterns (1). While yeast cells contain mostly high-mannose structures, mammalian-derived systems carry more complex glycans that include galactose, fucose and sialic acids (**Figure 1**)—all dramatically affecting the pharmacodynamics and pharmokinetics of the drugs, most notable in glycosylated-antibodies (11–13). Higher levels of sialic acid at the tips of glycan chains generally improves serum half-life and stability of biotherapeutics (12, 14, 15), partly since in the presence of terminal sialic acid glycosylated-biotherapeutics are not recognized by liver asialoglycoprotein receptors (ASGR1) or mannose receptors (MR; CD206), thereby preventing their rapid removal from circulation (12, 16). In addition, the negative charge of sialic acids positively contribute to their thermal stability and solubility (17, 18). Monoclonal antibodies constitute a major class of biotherapeutics, and in many of these antibodies the functionality is directly regulated by the glycosylation on their Fc domain. All IgG antibodies are glycosylated at a conserved asparagine residue (Asn297) in the Fc region (19), and some are also glycosylated at their Fab region (20–22). The glycan on Asn297 site modulates the shape of the Fc domain in a way that it alters its ability to interact with various Fc receptors (10, 15, 20, 23, 24). Remarkably, IgG

Fc glycosylation is altered in pathological conditions such as autoimmunity (25), infection (10), and cancer (26–28), thereby modulating their effector functions (29). Interestingly, removal of the whole *N*-glycan revokes the ability of the Fc domain to interact with Fc receptors, thus Fc glycosylation is essential for the IgG effector functions (13, 30). The absence of fucose residues enhances antibody-dependent cellular cytotoxicity (31). In addition, higher presence of galactose promotes complement-dependent cytotoxicity, while decreased galactosylation leads to alternative complement cascade activation (32, 33). IgG antibodies with higher amount of terminal α2–6-linked sialic acids are recognized by DC-SIGN on dendritic cells, leading to anti-inflammatory activity (34, 35), while on the other hand activation of dendritic cells through antibody aggregates may induce immunogenicity and development of anti-drug antibodies (36). Aiming to optimize glycosylation properties, currently most biotherapeutics are produced in mammalian expression systems, with their ability to produce human-like glycosylation (1, 2, 37). Major efforts had been put into various methods for cell-glycoengineering to control antibody glycosylation (1, 35, 38–40), or to predict the glycosylation based on computational modeling (13, 38, 41–44).

Although humans and most other mammals have relatively similar glycosylation patterns, two major differences have been identified. Unlike most other mammals, humans lack the enzymes to synthesize the Galα1–3Galβ1–(3)4GlcNAc (αGal) epitope and the common sialic acid *N*-glycolylneuraminic acid (Neu5Gc) (45) (**Figure 1**). In addition to the inability to naturally express these sugar structures, all humans produce circulating antibodies against both antigens (45–49). In contrast to αGal, exogenous Neu5Gc can be metabolically incorporated into newly synthesized glycans and become presented on human cells (50, 51). Co-existence of Neu5Gc-containing epitopes and circulating anti-Neu5Gc antibodies have been suggested to exacerbate chronic inflammation-mediated diseases (52–57). This immune-conflict may be further complicated with exposure to Neu5Gc-containing biotherapeutics, bio-devices or xenografts. Indeed, recent studies have suggested that Neu5Gc-glycans have an enormous diversity (58–60), and predicted to be widely found on various approved and marketed biotherapeutics (2, 61), such as Cetuximab (61) and anti-thymocyte globulin (62–65). Although biotherapeutics provide effective treatment for a variety of clinical conditions, suboptimal efficacy and safety are major concerns for many of these products. Herein, we discuss the unique situation of Neu5Gc-containing biotherapeutics in the face of anti-Neu5Gc responses in humans, and the current knowledge on the effects of Neu5Gc on immunogenicity, efficacy, and safety of biotherapeutics.

Neu5Gc IS IMMUNOGENIC IN HUMANS

Sialic acids are 9-carbon α-keto acidic sugars usually present at the outermost part of glycans in animals (5, 66). The two most common sialic acids in mammals are *N*-acetylneuraminic acid (Neu5Ac) and its hydroxylated form, Neu5Gc. Conversion of CMP-Neu5Ac to CMP-Neu5Gc is catalyzed by the enzyme CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) that is inactive in humans (66). In contrast to all other mammals, humans cannot synthesize Neu5Gc due to irreversible mutation in the *CMAH* gene that occurred ∼3 million years ago, before the appearance of the genus *Homo* (67–70). Nevertheless, consumption of Neu5Gc-containing mammalian-derived products (e.g., red meat and dairy) results in uptake of Neu5Gc-glycoproteins through micropinocytosis (71–73) and metabolic incorporation of Neu5Gc epitopes into newly synthesized glycans (50, 56, 72–74). Thus, low levels of Neu5Gc are present in human tissues, mostly on endothelium and epithelium, and are known to accumulate in certain pathological conditions, mostly in cancer (52, 56, 71, 75).

This unique phenomenon results in presentation of foreign antigen in the context of self (Neu5Gc is replacing the self Neu5Ac on existing cellular glycans), termed "Xeno-autoantigen" (47, 57). Hence, Neu5Gc is foreign in humans and mediates production of a complex anti-Neu5Gc antibodies response, or "Xeno-autoantibodies" (47, 51, 57, 76). Neu5Gc is a 325 Dalton molecule and cannot by itself fill the paratope of an antibody, yet Neu5Gc-containing glycan-epitopes are highly diverse (58–60) and are recognized by polyclonal anti-Neu5Gc IgM, IgA, and mostly IgG antibodies that make up 0.1–0.2% of total circulating antibodies in humans (47, 49, 77–79). Anti-Neu5Gc antibodies in humans arise already in infants, soon after the introduction of dietary Neu5Gc (e.g., cow milk in baby formula, meat-containing grinded foods), and have been suggested to be induced through uptake of dietary Neu5Gc by non-typeable *Haemophilus influenzae* (NTHi) during infection in infants (80), and through micropinocytosis of Neu5Gc-glycoproteins into human cells followed by recycling into the cells surface glycoproteins and glycolipids (71–74). In fact, all healthy humans examined thus far had anti-Neu5Gc antibodies, although sometimes at low levels and with limited repertoires (47, 49, 78, 81). This antibody response against Neu5Gc can be higher in certain pathologies and may remain high for decades (82–84).

Studies in mice had suggested that the co-existence of Neu5Gc-glycans and serum anti-Neu5Gc antibodies may lead to immune-driven chronic inflammation, termed "xenosialitis," thereby exacerbating chronic inflammation-related diseases such as cancer, cardiovascular disease and autoimmunity (52, 53, 57, 84–86). For example, high anti-Neu5Gc IgG titers were shown to be associated with increased risk for colorectal cancer (84), which also fits the reported association of red meat consumption and higher carcinoma risk (55, 87–89). Similarly, in a human-like mouse model (*Cmah*-KO) high consumption of Neu5Gc resulted in an inflammatory phenotype, and together with circulating anti-Neu5Gc antibodies (in *Cmah*/*Ldlr*-DKO mouse model) resulted in increased atherosclerosis (52, 86, 90). These findings in mice fit the reported high risk of cardiovascular disease that is associated with consumption of red meat and processed meat (91, 92), although clear evidence in humans is still controversial, at least through *in vitro* studies on effects of anti-Neu5Gc antibodies on human endothelial cells that express authentic Neu5Gc levels (65). Neu5Gc and anti-Neu5Gc antibodies had also been suggested to participate in autoimmunity (54, 55, 93). Altogether,

this unique human-specific immune conflict could help explain the susceptibility to numerous chronic inflammation-related diseases, which conspicuously occur in humans (94). The consequences of Neu5Gc/anti-Neu5Gc responses in humans could potentially be further exacerbated by exposure to Neu5Gc-containing biotherapeutics, bio-devices, or xenografts.

Neu5Gc ON MARKETED BIOTHERAPEUTICS ASSOCIATED WITH THEIR PRODUCTION SYSTEM

Production of many biotherapeutics involves non-human mammalian cells, serum or serum-derived substances, thus are likely to contain some levels of Neu5Gc. Generalizations cannot be made since glycosylation properties, including Neu5Gc levels, are influenced by many factors during the production process. Yet, it is reasonable to assume the relative Neu5Gc levels in biotherapeutics according to their production systems (2). The most common platform for biotherapeutics is Chinese hamster ovary (CHO) cells (1, 2, 95). Several studies have suggested the presence of Neu5Gc on biotherapeutics produced in CHO cells, though in relatively low levels (2, 95, 96). Baby hamster kidney cells (BHK-21) are also often used for production of biotherapeutics and are expected to express low levels of Neu5Gc (2). By contrast, murine myeloma cell lines (e.g., NS0 and Sp2/0) are known to produce Neu5Gc at significantly higher levels (2, 97, 98). Drugs produced in animals (non-human mammals that are known to synthesize Neu5Gc intrinsically; e.g., cow, pig, goat, sheep, and rabbit) are also likely to contain Neu5Gc, since they were shown to express high amounts of Neu5Gc (50, 60). For example, antithrombin produced in goat milk and anti-thymocyte globulin derived from rabbit, are known to contain high levels of Neu5Gc (2, 62, 63). Similarly, Neu5Gc is also widely found in xenografts that are used for organ and tissue replacement in humans, as demonstrated with tissues derived from cows and pigs (99–102). These findings also prompted the generation of Neu5Gc-deficient animals as novel platforms (103–107).

Human cell lines represent the ideal production platform in terms of glycosylation properties, but their high risk of viral transmission and low protein yield make them less popular for production of biotherapeutics (37). Nevertheless, several products derived from human cells (HEK293 and HT-1080) have been approved in recent years (1). Utilization of these cells may become more common in the future, yet the presence of Neu5Gc in their products remains a significant concern, as it can also be metabolically incorporated from exogenous sources (i.e., from the growth media). Hence, even human cells can produce Neu5Gc-containing biotherapeutics if Neu5Gc is unintentionally supplemented in their growth media, for example through the addition of animal serum or serum-derived substances (2, 45). Although it was well-known that humans cannot express Neu5Gc, its immunogenic potential was under-rated for years, and accordingly its presence on biotherapeutics was largely disregarded. With the accumulating information about Neu5Gc and anti-Neu5Gc antibodies in humans, the presence of Neu5Gc on biotherapeutics should be re-evaluated. While the effect of Neu5Gc on biotherapeutics remains poorly characterized, several recent studies addressed possible consequences (61–65, 108–111), as described below.

EFFECTS ON SERUM ANTI-Neu5Gc IgG RESPONSES IN HUMANS

Treatment of human patients with Neu5Gc-containing biotherapeutics can significantly alter the pre-existing immune response against Neu5Gc, both quantitatively and qualitatively. Yet, some studies failed to show human immune response against Neu5Gc-containing biotherapeutics, as in the case of recombinant erythropoietin that was produced in CHO cells (96, 112). Of note, these conclusions were based on the human response evaluated against Neu5Gc-containing gangliosides. It is possible that with current technologies as glycan microarrays it would be possible to revisit these findings. More recent studies were able to clearly demonstrate immunological effects in humans. Anti-thymocyte globulin (ATG) is an immunosuppressive biotherapeutic commonly used in transplantation and autoimmune diseases (113). ATG is a polyclonal IgG produced in rabbits and was shown to contain Neu5Gc (62, 63). One of the side effects during treatment with this drug is the development of an immune reaction against the non-human animal-derived immunoglobulins. This is characterized by immune complex formation that can develop into a serum sickness disease (62, 114). In fact, without strong immunosuppression most patients will develop serum sickness (114). Furthermore, it was shown that ∼10% of first-kidney graft recipients treated with the immunosuppressive drug ATG developed serum sickness disease, and in addition had increased serum anti-Neu5Gc IgG responses (62). The serum sickness was associated with late graft loss, and these patients exhibited further elevated titers of anti-drug and anti-Neu5Gc IgG in blood samples >4 years post-transplantation compared to patients without serum sickness (62). In another study, ATG treatment was found to be associated with a shift in anti-Neu5Gc IgG repertoire in transplantation patients over time (64). Similarly, analyzing early events in another prospective study of kidney-graft recipients within their first year showed that patients with ATG induction treatment had a highly significant increase in anti-Neu5Gc IgG levels compared to patients not treated with ATG. In addition, these antibodies shifted their response repertoire over time to recognize different Neu5Gc-glycans, even in the face of a strong immunosuppression in those patients, but no effect on the graft function was observed within the limit of this study (110).

While mostly used in transplantation, ATG therapy was also explored as a therapeutic drug in young adults within the Study of Thymoglobulin to arrest Type 1 Diabetes (START clinical trial) (114). In these diabetic patients, ATG treatment also resulted in a highly significant increase in levels of serum anti-Neu5Gc IgM and IgG that peaked after 1 month and remained detectable even 1 year after treatment (108). Further characterization of the top responders by elaborated glycan

microarrays demonstrated the rapid increase in responses against multiple Neu5Gc-glycans after 1 month, persistence over 2 years, and further demonstrated altered repertoires of serum anti-Neu5Gc IgG (63). In fact, ATG treatment changed the pre-existing response to induce anti-Neu5Gc IgG of higher affinity with extended diversity. Interestingly, in some patients there was *de-novo* recognition of various Neu5Gc-containing glycan epitopes, including of Neu5Gc-glycans normally expressed on glycolipids that were not present on the ATG drug (63). Overall, these findings suggested that Neu5Gc-containing biotherapeutics are immunogenic reagents, and once injected into humans that already express circulating anti-Neu5Gc antibodies, act as triggers of extended immune responses. In fact, current data support their role as inducers of secondary anti-Neu5Gc immune responses. In some individuals possibly also triggering a recall of memory responses inducing antibody recognition of Neu5Gc-glycans that had not been presented on the drug.

ANTI-Neu5Gc ANTIBODIES IN DISEASE

It was postulated that such elevated anti-Neu5Gc responses could potentially increase Neu5Gc-mediated xenosialitis and chronic inflammation-mediated diseases, as cancer and atherosclerosis (53). High pre-existing total anti-Neu5Gc IgG levels measured by glycan microarrays were associated with increased risk of colorectal cancer (in a cohort of 71 colorectal cancer cases and matched controls of the EPIC-Norfolk cohort plasma samples) (84). However, based on a large cohort of ∼200,000 kidney allograft recipients, average anti-Neu5Gc IgG responses measured by EIA method did not show increased colon cancer risk in the ∼18% ATG-treated patients compared to those not treated with ATG (111). Of note, these studies evaluated different pools of blood anti-Neu5Gc IgG antibodies and measured by different methods: pre-existing antibodies by arrays (84) vs. drug-induced antibodies by EIA (111). Currently, different methods are available to measure anti-Neu5Gc antibody responses (49, 115), and there are clear differences between pre-existing vs. ATG-induced anti-Neu5Gc IgG (63, 65), that together could perhaps explain the different analysis outcome regarding cancer risk.

Likewise, contradicting reports exist regarding anti-Neu5Gc antibodies in the context of cardiovascular disease risks. Aiming to examine gene expression profiles by *in vitro* studies, human endothelial cells that were engineered to express low levels of surface Neu5Gc (mimicking the levels expected to be present from dietary intake in humans) were exposed to different pools and dose of affinity-purified anti-Neu5Gc antibodies. This analysis showed differential gene expression when cells were exposed to ATG-induced compared to pre-existing anti-Neu5Gc antibodies or in the absence of such antibodies. Interestingly, drug-induced anti-Neu5Gc antibodies did not significantly upregulate inflammation-related genes that would be expected in xenosialitis (65). However, other *in vivo* studies in the human-like Neu5Gc-deficient mice also lacking the LDL receptor showed increased atherosclerosis propensity only when both high levels of diet-derived Neu5Gc-antigens and induced anti-Neu5Gc antibodies were present, thus supporting xenosialitis (90). Altogether, these findings suggest that anti-Neu5Gc antibody responses in humans are complex and further studies are needed to better understand their relationship with various diseases in humans.

RAPID CLEARANCE OF Neu5Gc-CONTAINING BIOTHERAPEUTICS *IN VIVO*—EVIDENCE IN MICE

Besides the immunogenicity of Neu5Gc on biotherapeutics, it was postulated that these Neu5Gc-drugs could potentially be recognized by circulating anti-Neu5Gc antibodies in humans, and by that affect drug levels and/or efficacy in patients. This was directly investigated using the top selling cancer biotherapeutic monoclonal antibodies targeting EGFR (61).

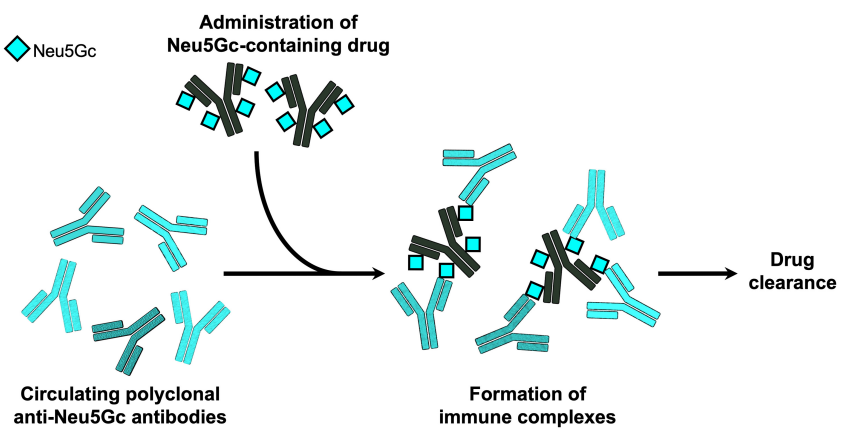

FIGURE 2 | Immune complexes of Neu5Gc-containing biotherapeutics. In the human-like Neu5Gc-deficient *Cmah*-KO mice, it was demonstrated that circulating polyclonal anti-Neu5Gc antibodies can bind Neu5Gc-containing biotherapeutic monoclonal antibodies and generate immune complexes that mediated rapid clearance of the biotherapeutic drug (61).

Consistent with their production system, it was shown that Cetuximab produced in murine myeloma cells contains Neu5Gc, while Panitumumab expressed in CHO cells lack Neu5Gc (61). Human serum anti-Neu5Gc antibodies could bind the Neu5Gc-containing Cetuximab and generate immune complexes, but did not bind Panitumumab. Furthermore, consistent with their expected immunogenic properties, injection of these drugs to the human-like Neu5Gc-deficient *Cmah*-KO mice induced serum anti-Neu5Gc antibody only in the Neu5Gc-containing Cetuximab-treated group. In these mice, circulating serum anti-Neu5Gc antibodies resulted in a rapid clearance of the Neu5Gc-containing Cetuximab, but not of Panitumumab (61). Together, these data suggest that Neu5Gc on biotherapeutics could potentially affect drug levels in circulation through immune complex formation (**Figure 2**), at least in human-like mice. Currently, there is no evidence of drug neutralizing activity of anti-Neu5Gc antibodies. It remains to be investigated whether Neu5Gc/anti-Neu5Gc could affect drug clearance in patients, hence alter drug efficacy and as such play a role in the variability of the clinical responses observed across a population for a given biotherapeutic.

CONCLUSIONS AND PERSPECTIVE

Biotherapeutics have revolutionized the treatment for numerous clinical conditions, yet immunogenicity and efficacy issues remain to be addressed. Currently, most biotherapeutics are produced in non-human mammalian cells to allow human-like glycosylation, as it was shown to dramatically affect pharmacological properties of these products. Yet, despite the fact that it was recognized that humans cannot produce the non-human carbohydrate Neu5Gc, its immunogenic potential was much ignored, and accordingly its expression on biotherapeutics was largely overlooked. In fact, non-human mammals produce Neu5Gc-glycans, against which all humans have circulating polyclonal antibodies. Moreover, Neu5Gc can be metabolized by human cells and become presented on cell surface glycans, glycoproteins and glycolipids. In addition, all humans examined thus far had serum anti-Neu5Gc responses at variable levels and repertoires. Neu5Gc on biotherapeutics may induce the pre-existing anti-Neu5Gc responses in humans, and these could potentially contribute to increased xenosialitis and related diseases, yet further evidence is needed to fully understand the developed responses and their effects in humans. Drug-induced or pre-existing anti-Neu5Gc antibody responses could potentially contribute to drug clearance from circulation through immune complex formation, thereby reducing drug efficacy, although clear evidence in humans is yet to be provided. While not discussed in this review, similar effects could be expected by αGal glycosylation on biotherapeutics since all humans have circulating anti-Gal antibodies. Thus, much of the mechanistic insights into the outcome of the co-existence of anti-Neu5Gc antibodies and antigenic Neu5Gc-containing biotherapeutics (or anti-Gal antibodies and antigenic αGal-containing biotherapeutics) in humans is largely unknown and warrants further investigation.

AUTHOR CONTRIBUTIONS

SY and VP-K wrote the manuscript.

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REVIEW
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The Possible Role of Anti-Neu5Gc as an Obstacle in Xenotransplantation

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Seventy to ninety percentage of preformed xenoreactive antibodies in human serum bind to the galactose-α(1,3)-galactose Gal epitope, and the creation of Gal knockout (KO) pigs has eliminated hyperacute rejection as a barrier to xenotransplantation. Now other glycan antigens are barriers to move ahead with xenotransplantation, and the N-glycolyl neuraminic acid, Neu5Gc (or Hanganutziu-Deicher antigen), is also a major pig xenoantigen. Humans have anti-Neu5Gc antibodies. Several data indicate a strong immunogenicity of Neu5Gc in humans that may contribute to an important part in antibody-dependent injury to pig xenografts. Pig islets express Neu5Gc, which reacted with diet-derived human antibodies and mice deleted for Neu5Gc reject pancreatic islets from wild-type counterpart. However, Neu5Gc positive heart were not rejected in Neu5Gc KO mice indicating that the role of Neu5Gc-specific antibodies has to be nuanced and depend of the graft situation parameters (organ/tissue, recipient, implication of other glycan antigens). Recently generated Gal/Neu5Gc KO pigs eliminate the expression of Gal and Neu5Gc, and improve the crossmatch of humans with the pig. This review summarizes the current and recent experimental and (pre)clinical data on the Neu5Gc immunogenicity and emphasize of the potential impact of anti-Neu5Gc antibodies in limiting xenotransplantation in humans.

Keywords: sialic acid, xenotransplantation, anti-Neu5Gc, graft rejection, pig, human disease, animal model

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INTRODUCTION

Organ transplantation is the treatment of choice for end-stage organ failure, but there are not enough human donors to transplant everyone who could benefit. In the United States alone, today more than 110,000 patients are on the United Network for Organ Sharing transplant waitlist, and just over 39,000 patients are transplanted each year (1). In Europe, in 2017, more than 144,000 patients are on waiting list, while almost 43,000 patients are transplanted per year (2). Many patients die before an organ becomes available, while others are never put on the list because they are too sick to wait for a donor organ. Xenotransplantation using pig organs could solve this shortage, but progress toward the clinic has been limited because humans possess antibodies to pig cells that trigger rejection of the graft immediately following graft reperfusion. One major obstacle of

Abbreviations: ADCC, Ab-dependent cell-mediated cytotoxicity; AMR, antibody-mediated rejection; ATG, anti-thymocyte globulin; CMAH, cytidine monophosphate N-acetylneuraminic acid hydroxylase; DSA, donor-specific antibodies; Gal, galactose-α(1,3)-galactose; GGTA1, α-1,3-galactosyltransferase enzyme; KO, knockout; mya, million years ago; Neu5Gc, N-glycolyl neuraminic acid; NHP, non-human-primate; NPCCs, Neonate pig pancreatic islets or islet-like cell clusters; PBMCs, peripheral blood mononuclear cells; WT, wild-type.

xenotransplantation is the rejection of the graft, often obtained from pig, the major candidate for xenotransplantation, by preformed and elicited anti-pig antibodies. The development of cloning for pigs coupled with the advances in targeted genome editing have made it possible to create pigs devoid of xenoantigens to which the xenoreactive antibodies bind (3–6).

A newborn human has few xenoreactive antibodies at birth, but by the first few months of age they have developed a mature xenoreactive antibody repertoire (7, 8). The first xenoantigen to be deleted in the pig was the α-1,3-gal (Gal) glycoprotein epitope that was produced by the α-1,3-galactosyltransferase enzyme (GGTA1) in pig cells (9). Since humans (and Old World monkeys) have deleted this gene during the course of evolution, they do not produce Gal (10, 11), and so they produce antibodies against this epitope when they encounter it during bacterial colonization of their gut during infancy (12). It has been suggested that pathogen bacteria, parasites, vector-borne pathogens and heat stable α-Gal-containing proteins in mammalian meat are other source of anti-Gal immunization in humans [reviewed in (13, 14)]. A Gal antigen is also synthesized in the globosphingolipid metabolism by the isoglobotrihexosylceramide 3 synthase (iGb3S, also called alpha-1,3-galactosyltransferase 2 [gene name *A3GALT2*)]. This enzyme is not functional in human (15). Both pig and mouse express the glycolipid form of the Gal epitope, which is less immunogenic and less recognized by human anti-Gal antibodies than the glycoprotein form (16–18).

Roughly 70–90% of the antibodies that humans have against the pig bind to the Gal epitope (19). The creation of the Gal knockout (KO) pig was a real advance for xenotransplantation, eliminating hyperacute rejection and improving kidney transplant survival in the pig-to-non-human primate (NHP) from hours to 6–16 days using clinically available immunosuppression. Immunopathological analysis of the rejected kidneys showed that AMR was still the cause of graft failure, suggesting that there were other xenoantigens that needed to be deleted to overcome the humoral barrier to clinical application (20). Despite the addition of human complement regulatory protein and thromboregulatory protein transgenes into Gal KO pigs, graft failure is nearly always secondary to AMR, suggesting that the humoral barrier will remain problematic until such time that it is eliminated through genetic engineering (21–23). Moreover, pre-existing antibodies present in human serum bind to Gal KO pig cells or tissues (24), confirming that non-Gal antigens have to be considered in the xenorejection (25, 26). The N-glycolyl neuraminic sialic acid (Neu5Gc or Hanganutziu-Deicher antigen) is a major sugar xenoantigen contained in glycoproteins and ganglioside glycolipids. In 2002, Alex Zhu et al. identified, using an hemagglutination array, Neu5Gc, as a non-Gal crucial xenoantigen (27).

NEU5GC, A MAJOR NON-GAL GLYCAN XENOANTIGEN

During the course of evolution [about 3 million years ago (mya) (28)], humans have lost the expression of functional CMAH, which is responsible for the hydroxylation of Neu5Ac (the Neu5Acetylated form of the neuraminidic acid) to create Neu5Gc. The theory is that absence of the CMAH conveyed protective immunity to a prevailing strain of malaria several million years ago (<3.5 mya), so that now humans do not produce Neu5Gc, rather they have Neu5Ac exclusively (19, 29). Consequently, Neu5Gc is seen as foreign by the human immune system when exposure occurs. Contrary to Old World monkeys, New World ones present also a *CMAH* gene inactivation 30 mya but independently from human beings (30). Thus, contrary to Gal KO graft that is assessable in Old World NHP that do not express the GGTA1 (20, 25, 31–33), and as only the New World monkeys and humans exhibit a lack of CMAH expression, the pig-to-NHP animal model is not conclusive to study the immunogenicity and the deleterious effects of anti-Neu5Gc antibodies on vascularized or cellular xenografts and may not provide a direct translation to the clinic.

Exogenous Neu5Gc is incorporated into cell surface lipooligosaccharides of non-typeable *Haemophilus influenzae* in the nasopharynx of humans. Neu5Gc from food is taken up by non-typeable *Haemophilus influenzae*, and humans form antibodies against Neu5Gc as well as against non-typeable *Haemophilus influenzae* (34). Other bacteria of the microbiota have the capacity to take the Neu5Gc from food and to use it as carbon source (35) but their role in inducing an anti-Neu5Gc humoral response is not known. Other human main natural exposure to Neu5Gc comes from diet directly (mammalian meat, especially processed and industrial forms, milk, cheese . . .). In this case, small quantities of ingested Neu5Gc seem to be absorbed and deposited in low amounts on human epithelial and endothelial cells (36, 37). After micro-pinocytosis by human cells, Neu5Gc is integrated into various glycans and glycolipids, and is then expressed on the cell surface (37–39).

In the context of xenotransplantation, Neu5Gc is largely detected on non-human mammal epithelial cells and accumulates on endothelial cells (38–41). Although the identification of the Neu5Gc antigen has not been as detailed as in other animal species such as mice, rabbits, sheep and cattle, pig expresses the CMAH and Neu5Gc antigens. In wild-type (WT) pigs, the Neu5Gc/Neu5Ac ratio varies in tissues depending on the CMAH activity intensity, but Neu5Gc is thus present in pig heart, kidney, spleen, lung, cornea and liver (42–45). The antigenicity of NPCCs (46) and adult porcine islets (47–49) was also linked to the expression of Neu5Gc epitopes and the presence of Gal antigen (50). Neu5Gc is also largely detected in erythrocytes (51). Pig leukocytes (mainly lymphocytes) released during the perfusion of vascularized pig organ may contribute to xenograft recipient immunization as these cells exhibit the major Neu5Gc-GM_3 and -GD_3 gangliosides together with Gal-terminated compounds (52). Invalidation of the gene encoding for the GGTA1 seems to increase the expression of Neu5Gc gangliosides and antigens (43, 53), and produces a raise and diversification of acidic glycolipid-specific antibodies after transplantation of a Gal KO heart into baboon (43). In this last study, these anti-acidic glycolipid induced antibodies are very probably not specific for Neu5G since the baboons express this antigen like the pig. However, we can suspect that a large anti-acidic xenoantigen response including

anti-Neu5Gc will increase in human in a similar situation. Moreover, mutating the *CMAH* gene together with the *GGTA1* gene reduces antibody binding of almost all human serum tested compared to *GGTA1* KO (54, 55). Finally, despite a high concentration of Neu5Ac in the brain of vertebrates, Neu5Gc is detected at very low level in mammal brain, concentrated in endothelial cells but absent in neurons, probably in line with a neuronal down regulation of the expression of the CMAH encoding gene (56).

Neu5Gc IMMUNOGENICITY

Diet-Derived Anti-Neu5Gc in Humans

At least 80% of humans possessed anti-Neu5Gc antibodies in a similar level of anti-Gal antibodies (27, 57). Following exposure to dietary Neu5Gc, anti-Neu5Gc IgG and IgM develop in infants (34). As anti-Gal (58), anti-Neu5Gc antibodies are of the IgA, IgM and IgG isotypes (59) but with a predominance of IgG for anti-Neu5Gc antibodies (27, 59). Contrary to anti-Gal antibodies that are detected at high level in all individuals, anti-Neu5Gc antibodies are found at variable levels (59, 60) and undergo affinity maturation during life (61). These differences could be in line with the putatively lower antigenicity of Neu5Gc than this of Gal. Indeed, in contrast with anti-Gal antibodies that are produced similarly to humans by *Ggta1*$^{-/-}$ mice (62), even if at lower levels (63), and by *GGTA1*$^{-/-}$ pigs (64–66), anti-Neu5Gc are not detected in *Cmah*$^{-/-}$ mice even feed with Neu5Gc carrying food, and could be induced only by a strong immunization with Neu5Gc-loaded non-typeable *Haemophilus influenza* (34), non-microbial Neu5Gc (67) or Neu5Gc positive xenogeneic cells (68), suggesting that anti-Neu5Gc induced in *Cmah*$^{-/-}$ mice are more related to elicited antibodies than diet-derived ones. Besides, many Neu5Gc epitopes on various glycoproteins and glycolipids (69) are targeted by anti-Neu5Gc antibodies, which was not the case of anti-Gal ones that recognized a dominant Gal (Galα1-3Galα1-(3)4GlcNAc-R) epitope on glycoproteins (70). Also, contrary to Gal glycolipids, Neu5Gc glycolipids are recognized by human xenoreactive antibodies (16, 17).

High Titers of Anti-Neu5Gc Antibodies Elicited in Humans by Animal-Derived Medical Antibody and Biodevice Exposure

The xenograft survival can depend not only on the presence of diet-derived anti-Neu5Gc antibodies, but also and more particularly on the presence of anti-Neu5Gc antibodies elicited at high titers previously in the same patient by animal-derived therapies prior to xenotransplantation. Indeed, beside bacteria and diet, animal-derived biodevices and immunotherapies expose humans to a larger amount of Neu5Gc and produce high titers of anti-Neu5Gc antibodies. Anti-Neu5Gc antibodies were first identified in patients who had been exposed to animal serum, identifying Neu5Gc as a xenoantigens prior to its evaluation as a barrier to clinical xenotransplantation. Purified animal immunoglobulins express Gal and Neu5Gc in the variable Fab region and linked to Asn297 of their Fc domain (71). Rabbit IgG display Neu5Gc, but no Neu5Ac (57). Anti-Neu5Gc IgM and IgG quantified by ELISA toward a large panel of Neu5Gc epitopes (72) increase significantly with a peak at 1 month post-treatment in the serum of non-immunosuppressed patients treated with rabbit polyclonal ATG (73) (**Table 1**). However, all these patients do not exhibit anti-Neu5Gc antibodies and anti-Neu5Gc IgG were induced vigorously in about 20% of the patients. Glycan microarray shows that pre-existing anti-Neu5Gc IgG rapidly diversify their repertoire of recognition of Neu5Gc epitopes on glycoproteins and glycolipids including new Neu5Gc epitopes not recognized before rabbit ATG administration (74) (**Table 1**). In immunosuppressed patients, Neu5Gc on rabbit ATG is also antigenic and elicits a higher and diversified anti-Neu5Gc humoral response within the first year post-kidney allotransplantation (57, 75) (**Table 1**).

Another potential sources of Neu5Gc can come from treatments with biologicals (including monoclonal antibodies, recombinant virus and proteins) produced in CMAH-expressing cells such as CHO cells or other non-human mammalian cells (76). The Cetuximab humanized murine antibody has been implicated in anti-Gal IgE dependent anaphylaxis in some treated patients (77) and exhibit Neu5Gc epitopes that induce anti-Neu5Gc antibodies in the *Cmah*$^{-/-}$ mouse (78). Neu5Gc glycosylations are also detected in Erythropoietin produced in CHO (79, 80). In addition, Neu5Gc derived from animal components and present in the medium can also be incorporated into glycans present on recombinant products.

Pig (and bovine) prosthetic heart valves that are devitalized and decellularized to be less immunogenic for clinical use can have long-term successful function in humans. However, despite the cleaning by glutaraldehyde of all pig cells in these devices, residual Gal and Neu5Gc antigens are detected by using immunohistochemistry, HPLC and mass spectrometry (81–83). Anti-Gal (84) and anti-Neu5Gc IgG detected by using sialoglycan microarrays are elicited after implantation of these valves in humans (81, 85, 86). In this way, these anti-Neu5Gc antibodies bind to pig and bovine valves and commercial valves (81). Valves from *GGTA1/CMAH* KO pigs have a reduced human IgM and IgG binding compared to WT pig valves (85). Anti-Gal antibodies have been shown to mediate an inflammatory calcification of bioprosthetic heart valves (87, 88) and have been implicated in their deterioration (89, 90). It is possible that anti-Neu5Gc that recognized various epitopes on bioprosthetic heart valves could participate to their deterioration too.

The study of anti-Neu5Gc humoral response in patients treated with engineered porcine skin dressings confirms the high immunogenicity of Neu5Gc in humans (91, 92) (**Table 1**). Linda Scobie et al. examined the serum of 220 burn patients who received pig skin grafts for dermal coverage and found that, beside a moderate enhancement of anti-Gal antibodies, Neu5Gc glycans appear to be major non-Gal xenoantigens recognized by anti-Neu5Gc IgM and IgG that remained elevated in patient's serum for years (34 years was the longest follow up) (92). Serum from burn patients did not show significant binding to Neu5Ac glycans but exhibit a large preference to N-linked glycans

TABLE 1 | Anti-Neu5Gc antibody in Neu5Gc non-concordant xenosituation.

	Anti-Neu5Gc detection method	Delay post-graft	Anti-Neu5Gc pathogenic potential impact	References
Animal-to-Human				
Pig fetal islet–like cell clusters	Glycan array	1 to 12 months	N/A	(101)
Pigskin	ELISA, glycan array	Up to 34 years	N/A	(92)
Equine, Rabbit anti-thymocyte globulin	ELISA, glycan array		Cytokine release syndrome and serum sickness	(57, 73–75, 113, 119)
Pig-to-Neu5Gc KO mouse				
Neonate pig islet in the *Cmah*$^{-/-}$ mouse	Flow cytometry	7 days	N/A	(102)
Rodent-to-Neu5Gc KO mouse				
Neu5Gc^{+} thymocytes in the *Cmah*$^{-/-}$ mouse	Flow cytometry	1 to 4 weeks	*In vitro* cytotoxicity	(68)

(Neu5Gcα2-6LacNAc/Lac) and to a lesser extent to O-linked glycans (Neu5Gcα2-3Core1).

All these results confirm that induced anti-Neu5Gc antibody represents a major immune response in xenotransplantation.

LESSONS FROM XENOGRAFTS IN HUMAN PATIENTS

In addition to the treatment with animal immunoglobulins and dressings with pig skin, xenografts have been done in humans and represent situations of mismatch for Neu5Gc.

NHP-to-Human

Early modern attempts at clinical xenotransplantation used non-human primates as donors of kidney, heart and liver. NHP and humans have both lost the capacity to synthetize Gal. NHP graft in humans are then fully concordant for Gal. Since NHP have a functional *CMAH* gene contrary to humans and produce Neu5Gc (93), humans have a positive crossmatch to the NHP (55) and it is possible that anti-Neu5Gc antibodies were contributors to graft loss. A (hyper)acute or chronic antibody mediated rejection associated with a transplant dysfunction was described as the cause of graft failure in some few NHP-to-Human cases (94–99). However, the antibody studies in the human recipients of NHP xenografts are rather scant and data for antibodies specific for Neu5Gc are lacking, making the role of these antibodies somewhat speculative in these NHP-to-Human graft failures. In the NHP kidney-to-Human situation precisely analyzed by Apolline Salama and Jean-Paul Soulillou in a recent review (29), most (19 among 21) of the NHP kidneys grafted in humans are functional for at least 10 days and until 9 months, suggesting that, pre-existing in recipients, anti-Neu5Gc antibodies, if implicated in rejection, have a less detrimental effect on the vasculature of the graft than the anti-Gal antibodies could have had in the Pig-to-Human situation but which could not be directly involved in NHP-to-Human situation.

Pig Pancreatic Islet Xenotransplantation

In the context of pig islet xenografts in humans, donors and recipients are not concordant for both Gal and Neu5Gc. Gal antigen is weakly expressed by adult porcine pancreatic islets, but exhibits a high level of expression on newborn pig islets (50), justifying the editing of the *GGTA1* gene at least for the use of NPCCs in future clinical trials. The expression of the Neu5Gc antigen is detectable in neonatal and adult pig islets (46, 47, 100). The detection of anti-Neu5Gc antibodies in human serum is subject to specific difficulties including the diet, the molecular diversity of Neu5Gc epitopes, and the individual variability in anti-Neu5Gc isotypes and antigen recognition patterns (29). This could explain why no increase in anti-Neu5Gc antibody levels in humans following transplantation with fetal islet-like cell clusters has been detected when a unique Neu5Gc-GM3 antigen was used in the anti-Neu5Gc array (40). However, when a broad spectrum of glycan antigens was tested using a glycan array covering a lot of Neu5Gc different epitopes, anti-Neu5Gc antibodies could be clearly detected in some patients grafted with fetal islets but in a less extent than anti-Gal antibodies (101) (**Table 1**). Pig islet Neu5Gc and sialic acid antigens were implicated *in vitro* in complement-dependent injury of islets by human antibodies and contributed clearly to the antigenicity of pig islets (46, 47). In one study (100), the absence of Neu5Gc expression on isolated islet cells obtained from *CMAH* KO pig did not reduce human antibody binding. However, recent results indicated clearly that anti-Neu5Gc antibodies were produced in the humanized *Cmah*$^{-/-}$ mouse model following WT and Gal KO neonatal islet grafts (102) (**Table 1**).

DIET-DERIVED AND ELICITED ANTI-NEU5GC ANTIBODIES, A DIFFERENT CONCERN FOR THE PIG XENOGRAFTS IN HUMANS?

Pre-existing antibodies present at transplantation (i.e., antibodies following diet exposure and those previously elicited by another Neu5Gc contribution than xenotransplantation, like animal immunoglobulin treatment, skin or devitalized biodevices) would be expected to trigger, together with anti-Gal antibodies, the rejection of a WT pig xenotransplant. The high titer of anti-Neu5Gc circulating antibodies generated by the xenotransplantation in an individual (probably similar to those elicited by prior medical treatments) is likely (1) to drastically reduce the survival of the graft containing Neu5Gc and (2)

to increase the risk in recipient of chronic inflammation and of associated diseases such as carcinomas and atherosclerotic vascular disease.

Diet-Derived Anti-Neu5Gc Antibodies

Antibodies specific for Neu5Gc are the majority among non-Gal-specific antibodies that could impact xenotransplantation (27, 54, 55, 59, 60, 103). Pre-existing anti-Neu5Gc IgG exhibit cell- and/or complement-dependent cytotoxicity toward human cells *in vitro* (104) and ADCC of Neu5Gc positive cells (105). Human sera exhibiting high-titer of general anti-Neu5Gc (and not those without) have been described to lead to endothelial cell activation, complement deposition, E-selectin expression, increased pro-inflammatory cytokine secretion, and altered monocytes functions (103, 106). Diet-derived antibodies specific for Neu5Gc may contribute to aggravate lesions caused by anti-Gal antibodies following a pig xenograft in humans (29).

In vascularized xenografts, similarly to (106) that used human endothelial cells exhibiting high Neu5Gc concentrations to describe the vascular effect of diet-induced anti-Neu5Gc antibodies, Neu5Gc positive endothelium from WT pig could be targeted immediately by diet-derived anti-Neu5Gc antibodies that could induce the degradation of the xenograft function (103). In line with this data, anti-Neu5Gc antibodies described to be able of ADCC reactions (107) may participated to the immediate lyse of WT pig endothelial cells (108). Anyway, the production of anti-Neu5Gc antibodies induced by the WT xenograft will quickly take over the effects of diet-derived anti-Neu5Gc antibodies. For Neu5Gc KO xenograft, however, we cannot exclude local inflammation of the graft after colonization by the endothelial cells of the human recipient loaded by diet-derived Neu5Gc.

Elicited Anti-Neu5Gc Antibodies

Evoked anti-Neu5Gc antibodies (**Table 1**), which persist for a long time and are characterized by a diversification of their recognition repertoire, could mediate biological effects different from pre-existing antibodies. The transcriptome of human endothelial cells exhibiting "physiological" amounts of Neu5Gc is differentially affected by diet-derived and elicited anti-Neu5Gc antibodies (109). Of note, the involvement in xenotransplantation of elicited antibodies specific for Neu5Gc will be probably different depending on the individual as animal products, cells, tissues and organs do not induce anti-Neu5Gc antibodies in all treated humans and these antibodies show an individual-related variable affinity and specificity for the multiple Neu5Gc-containing antigens (74, 75, 92, 101, 110).

In vitro, anti-Neu5Gc antibodies from immunized $Cmah^{-/-}$ mice are implicated in the complement-mediated lysis and ADCC of Neu5Gc positive cells (68) (**Table 1**). The loss of Neu5Gc during evolution promoting inflammatory macrophages and phagocytosis (111), can thus impacts xeno-tissue and -organ rich in macrophages such as the lung. "Humanized" $Cmah^{-/-}$ mouse model that develops elicited anti-Neu5Gc antibodies following Neu5G-positive thymocyte immunization rejects WT Neu5Gc-positive syngeneic islets (68). Since the only difference between donor and recipient was the absence of the *Cmah* gene in the recipient, anti-Neu5Gc antibodies were participants in early graft failure in the murine recipient, allowing to anticipated that it will be the same in humans as almost all-human IgG that bind non-Gal pig antigens are shown to be anti-Neu5Gc and responsible for determinant injury to Gal KO cells and organs (54).

Surprisingly, Neu5Gc KO mice do not reject a murine Neu5Gc positive heart while a Gal KO mouse rejects a mouse heart expressing Gal, suggesting that, in some situations such as perhaps a vascularized graft, the anti-Neu5Gc may add to the anti-Gal response in the hyperacute rejection but is not sufficient alone (68). Today, further investigations on the role of anti-Neu5Gc antibodies are necessary to allow a better comprehension of their potential deleterious role in xenograft survival. It is not clear whether anti-Neu5Gc antibodies could be always implicated in delayed xenograft rejection neither in NHP that express Neu5Gc, nor in humans because of the difficulty to establish a cause-and-effect relationship.

In human recipients, we can suspect that elicited anti-Neu5Gc antibodies may induce or participated to chronic inflammatory phenomena such as chronic vascular inflammation (xenosialitis) and tumor progression (37), owing to the presence of diet-derived Neu5Gc epitopes on endothelial cells (57, 106) and some epithelia (37, 112), creating thus a condition of *in situ* immune complex disease (57). Even in the presence of immunosuppression, anti-Neu5Gc antibodies are evoked and may have deleterious effects (57, 75) but their presence in (non-immunosuppressed) ATG-treated patients does not seem to produce clinical sign of vascular injury (113). As the patterns of glycosylation differ between normal and tumor cells, Neu5Gc incorporated into tumor cells will be perceived as foreign neo-antigens by humans (114, 115), however, their contribution is discussed and appears complex (115, 116). Anti-Neu5Gc induced by animal biodevices and/or xenotransplants may also activate the recipient endothelium and provoke chronic lesions of the recipient own vasculature in several organs and tissues following binding of anti-Neu5Gc on endothelial cells and complement activation (106). Compared to sera without anti-Neu5Gc, human sera containing high titer of ATG-elicited anti-Neu5Gc antibodies induce an increase in transcripts (qRT-PCR) encoding for IL-1β, IL-6, and IL-8 pro-inflammatory cytokines in human endothelial cells (57). However, elicited anti-Neu5Gc antibodies do not seem to induce an inflammatory transcriptomic profile in human endothelial cells loaded with a "physiological" Neu5Gc concentration similar to this obtained from diet (109). It has been also described that Neu5Gc and anti-Neu5Gc antibodies may contribute to exacerbate atherosclerotic cardiovascular disease mediated by xenosialitis (37, 106, 117), infectious mononucleosis (118), early serum sickness disease (57, 113, 119), and autoimmunity (120). This last acceptation may depend of the autoimmune disease considered, as multiple sclerosis seems to be more associated to the decrease of anti-Gal antibodies than to an altered titer in anti-Neu5Gc (121). The repertoire shift of elicited anti-Neu5Gc antibodies and their recognition with higher affinities of new epitopes and new Neu5Gc-containing glycans, may be more related to their deleterious implications than their

global titer increase (122) in local inflammation or serum sickness disease.

ELIMINATING NEU5GC AS A PROBLEM IN XENOTRANSPLANTATION

New nuclease-based genome editing tools, especially CRISPR/Cas9 have made it very straightforward to delete genes in pig cells to provide a direct evaluation of candidate xenoantigen (5, 123–125). Genes thought to produce xenoantigens can be deleted in an immortalized pig cell line and screened for the presence or absence of xenoreactive antibody binding (126). Neu5Gc was the first such xenoantigen to be tested and validated as a xenoantigens using the nuclease-based genome editing approach (54, 55).

Today, Gal KO pig hearts expressing human CD46 and thrombomodulin exhibit with success a more than 6 months orthotopic function in baboons (127). Immunofluorescence staining of the myocardium and plasma levels of non-Gal xenoantibodies does not indicate humoral rejection of the graft. The translation to the Pig-to-Human clinic situation may probably require the KO of Neu5Gc in donor pig, as the expression of CD46 may be insufficient even if it regulates complement molecules such as c3b and c4b that could be activated by the anti-Neu5Gc response (60).

Zing Finger and then Tale Nucleases were used to perform the first series of multiplex edits in pigs by deleting both GGTA1 and CMAH before using somatic cell nuclear transfer to create new

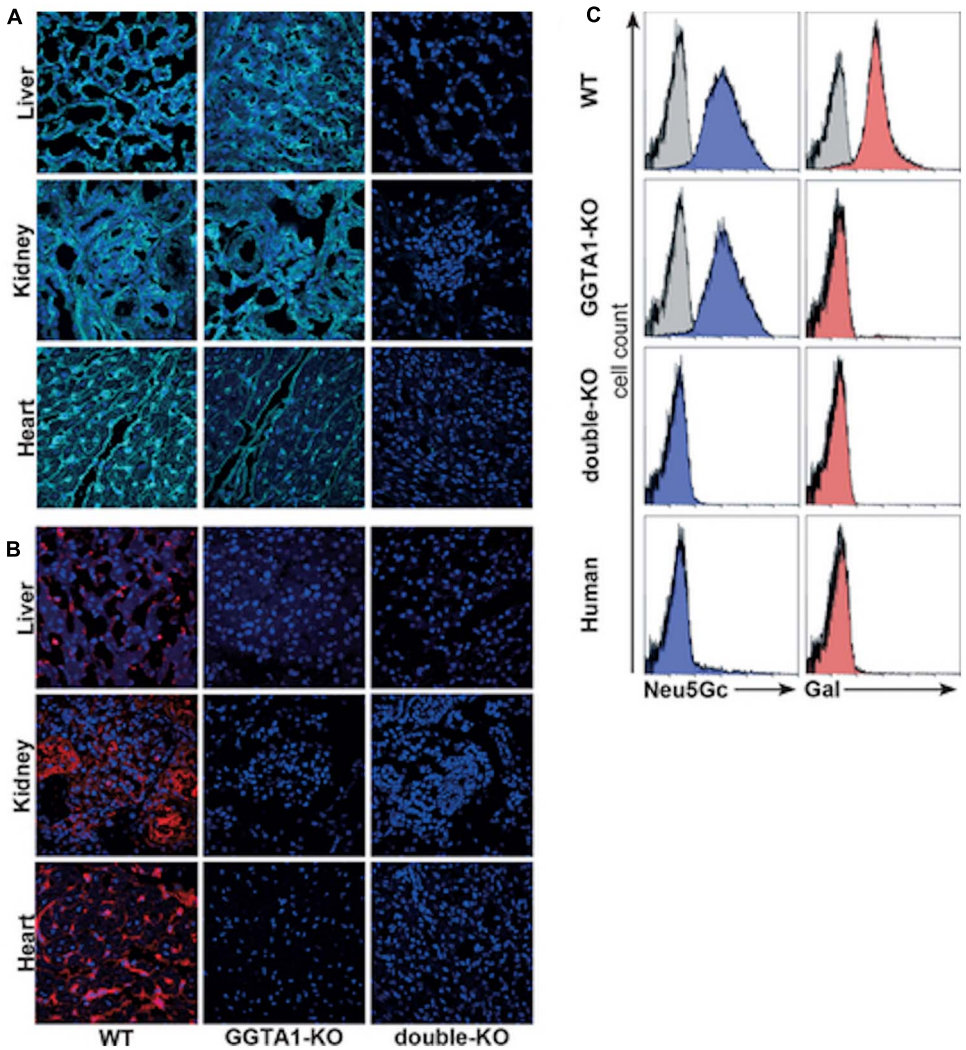

FIGURE 1 | Analysis of carbohydrate epitopes in genetically modified pigs (54). Confocal microscopy of 2-month-old WT, 8-month-old *GGTA1* KO, and 2-day-old *GGTA1/CMAH* double-KO porcine tissues stained with **(A)** anti-Neu5Gc chicken IgY (cyan, Sialix, Vista, CA, United States) and **(B)** IB4 lectin (red). **(C)** Flow cytometric analysis of PBMCs labeled with anti-Neu5Gc antibody (blue) and IB4 lectin (red). Unstained PBMCs were the negative controls for IB4 lectin, and an isotype negative control was used in the anti-Neu5Gc staining. Although shown, the negative controls are difficult to see in some panels because of overlap with the experimental group.

pigs (54, 102, 128–130). These pigs were important for progress in xenotransplantation, and particularly in the evaluation of Neu5Gc as a barrier to clinical implementation. The first finding that was important for xenotransplantation is that multiple engineering could be performed in pigs without compromising viability. This means that it is possible to make multiple modifications in a given pig so that it now takes 6 months to create a pig with many targeted gene edits using nuclease-based technology, as compared to 3 years to create a single homozygous edit to a single gene using homologous recombination and breeding (54, 55). Now with the development of CRISPR/Cas, multiplex engineering has become even simpler (5, 125, 131). The second finding that was just as critical was that the deletion of CMAH eliminated Neu5Gc in all pig tissues. Neu5Gc was expressed in all commonly transplanted tissues; heart, kidney, liver, pancreas, and lung (**Figure 1**). These pigs made it simple to definitively answer the question of whether Neu5Gc was a significant xenoantigen. Crossmatch results performed using human serum and *GGTA1/CMAH* KO pig cells in flow cytometric crossmatching assays demonstrated that the deletion of Neu5Gc reduced the serum content of IgG and IgM that bound to pig cells significantly. The reduction was significant enough that the crossmatch showed that the binding of xenoreactive antibodies to PBMCs was lower in the *GGTA1/CMAH* KO pigs than it was to chimpanzees (55) (**Figure 2**).

The immediate question is whether the additional reduction in xenoreactive antibody binding afforded by deleting CMAH on the *GGTA1* KO background has reduced the humoral barrier to the point where clinical implementation is appropriate. Evaluation of the crossmatches from patient serum with the

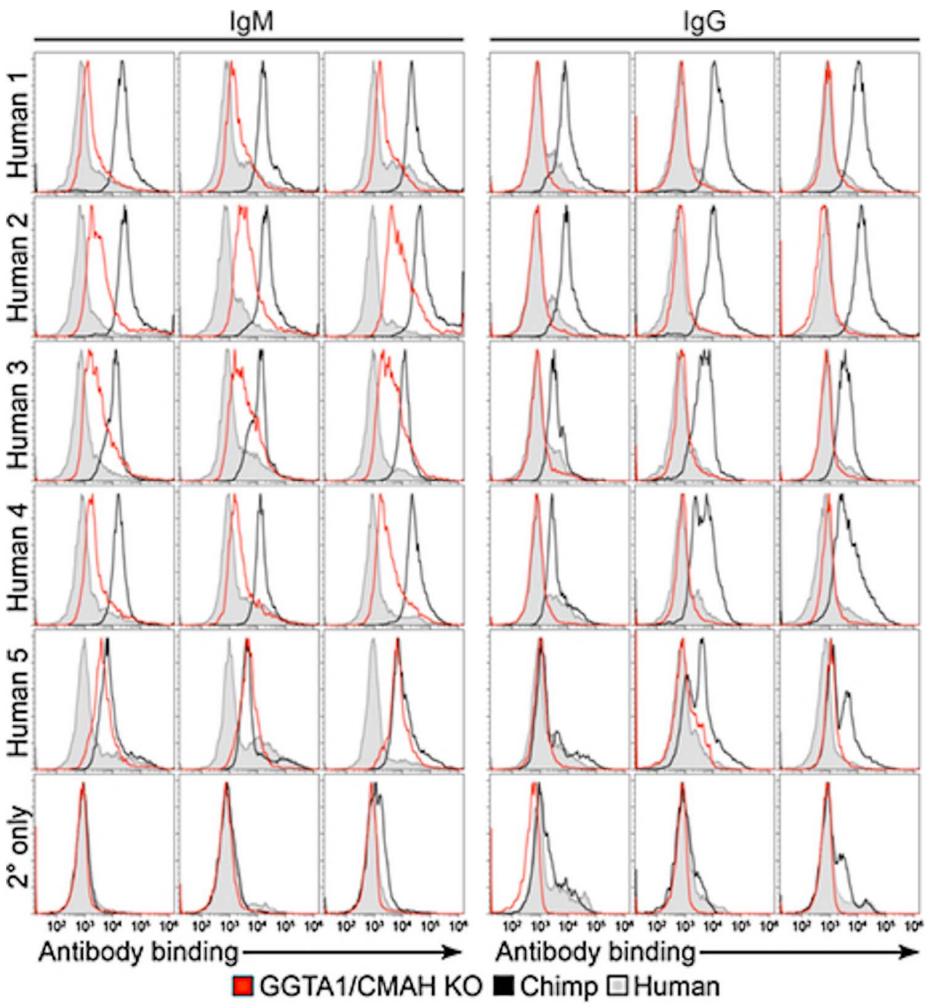

FIGURE 2 | Comparison of human antibody binding to human, porcine or chimpanzee cells (55). PBMCs isolated from humans, chimpanzees and *GGTA1/CMAH* KO pigs incubated with 25% serum collected from five humans. Levels of IgM or IgG binding were detected using fluorescently labeled anti-human IgM or IgG antibodies followed by flow cytometry analysis. Histogram profiles of human IgM and IgG antibody binding are shown for three humans (filled gray), three chimpanzees (black) and three *GGTA1/CMAH* KO pigs (red). Histogram profiles of PBMCs incubated with fluorescently labeled anti-human IgM or IgG antibodies in the absence of human serum are shown to indicate background fluorescence (2° only). CMAH, cytidine monophosphate-N-acetylneuraminic acid hydroxylase; GGTA1, galactosyltransferase.

GGTA1/CMAH KO pig PBMCs showed that xenoreactive antibody binding, while greatly reduced, was still significant enough that further xenoantigen reduction would be necessary (54, 55). The cellular dependent cytotoxic crossmatches were still positive, indicating that some degree of early AMR would be expected in the absence of any pre-transplant immunomodulatory therapy. Recently, the immunodominant glycan of the Sda antigen group (β1-4-linked GalNAc) has been identified as a significant xenoantigen in humans as well as Old World monkeys (132, 133). The *B4GalNT2* gene encodes for the β1,4 N-acetylgalactosaminyltransferase that catalyzes the terminal addition of GalNAc to a sialic acid modified Gal, producing thus the Sda antigens [reviewed in (134)]. Considering that the *B4GalNT2* gene remains functional in all humans excepted only in about 4% to 5% of humans that are Sda-negative, and that only about half of these Sda-negative individuals exhibit anti-human Sda antibodies especially of the IgM isotype (135–137), it was first predicted that GalNAc would not be an important xenoantigen in the Pig to-Human combination. Once *GGTA1/CMAH/B4GalNT2* KO pigs were created, it was clear that the Sda antigen was a xenoantigen. *GGTA1/CMAH/B4GalNT2* triple KO in pig reduce human antibody binding to pig PBMCs in a flow cytometry crossmatch to clinically acceptable transplant levels (MFI > 5000) in 80% of waitlist patients, 59% of waitlist patients have a complete negative crossmatch to IgG (MFI > 2000), and 31% of patients have a complete negative crossmatch to both IgG and IgM (MFI > 2000) (138). Accurate identification of donor specific pre-existing antibodies in recipient serum is critical to achieve the best post-transplant outcomes. Human IgM and IgG binding to Gal/Neu5Gc/GalNAc KO kidney endothelial pig cells was also significantly reduced compared to cells from Gal and Neu5Gc-deficient pig cells (124, 126). Immunofluorescent staining of Gal/Neu5Gc/GalNAc KO tissues incubated with human serum confirmed a decrease of human IgG and IgM binding in heart, lung and kidney tissues compared to WT pigs contrary to liver, spleen and pancreas of triple KO pigs that linked comparably human immunoglobulins than WT (42). However, in this semi-quantitative study, no comparison with Gal and Neu5Gc KO pig tissue was provided. Finally, anti-Sda IgM and IgG are elicited following blood transfusion of Sda-positive human erythrocytes (139) and may interfere with xenotransplantation.

CONCLUSION AND PROSPECTS

Naturally occurring anti-Neu5Gc are present in nearly all humans, and creation of Gal/Neu5Gc KO pigs has reduced human antibody binding to the point where the pig has a better crossmatch (fewer antibodies) than the Human-to-Chimpanzee combination. Anti-Neu5Gc (i.e., pre-existing diet-derived antibodies present at transplantation and those elicited by other Neu5Gc contribution than xenotransplantation like animal immunoglobulin treatment, skin or devitalized biodevices) may participate, together with anti-Gal antibodies, to the humoral rejection of the WT pig xenotransplant. Anti-Neu5Gc antibodies elicited by a WT pig graft contribute to the AMR of the graft and may aggravate inflammatory processes in the human recipient caused by the presence of diet-derived Neu5Gc on human cells.

Anti-Neu5Gc antibodies elicited by challenges with xenogeneic tissues or animal-derived immunoglobulins are able to reject pancreas islets in an experimental setting in the mouse. Elicited responses to newly recognized Neu5Gc antigens could be higher detrimental than the diet-derived anti-Neu5Gc responses, suggesting that xenografts lacking Neu5Gc would be safer for the transplant and for the human recipient. The consequences of long-standing exposure to high levels of elicited anti-Neu5Gc antibodies are not well documented and need today to be evaluated. Pig lines have been developed KO for the expression of Gal and Neu5Gc and now also including the KO for GalNAc, in order to eliminate the humoral barrier to clinical xenotransplantation for a great number of people.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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OPINION
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Challenging the Role of Diet-Induced Anti-Neu5Gc Antibodies in Human Pathologies

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INTRODUCTION

The thematic issue of *Frontiers in Immunology,* entitled "Human Antibodies Against the Dietary Non-Human Neu5Gc-Carrying Glycans in Normal and Pathological States," benefits from an extensive fundamental review of previous studies on N-glycolylneuraminic acid (Neu5Gc) in humans [(1) for recent review and citations therein] and from studies on anti-Neu5Gc antibodies (A-GcAbs) in *Cmah*$^{-/-}$ mice that, similar to humans, lack a functional cytidine monophosphate N-acetylneuraminic acid hydroxylase (CMAH), thereby allowing Neu5Gc synthesis (2). Further, "xenosialitis" assumes that the local interaction of A-GcAbs with traces of Neu5Gc residue at the surface of certain human cells may result in a chronic activation of Neu5Gc-displaying cells, which would eventually generate atheroma [for endothelial cells (ECs)] and malignancies (for epithelia) (1, 3). However, not many studies have been performed to date in humans, and these studies have not yet unambiguously demonstrated that A-GcAb-related xenosialitis could contribute to major diseases in humans.

This opinion article aims to assess the available evidence-based background of the concept of a deleterious effect of xenosialitis on human diseases, and to suggest alternative working hypotheses for future studies. As previously mentioned, xenosialitis, which is restricted to human pathologies in this opinion article, has been proposed as a "logical" basis for deleterious effects that would result from *in situ* interactions of A-GcAbs, which are present in human sera and diet-derived Neu5Gc deposits on certain human cells (1). However, this framework assumes the paradox that evolution would have allowed such a dangerous confrontation. Results from studies on *Cmah*$^{-/-}$ mice have been extensively reviewed (1). However, using CMAH-deficient mice—which do not benefit from the coevolution adaptation that followed the lack of Neu5Gc in humans— poses an issue, as xenosialitis models require exogenous immunizations to *elicit* A-GcAbs (4, 5), due to the difficulty of "humanizing" the mice with a Neu5Gc-rich diet. In addition, cases of "positive data" arguing for xenosialitis in animal models have required experimental designs that use mice which are prone to spontaneously developing endothelial injuries (5) [see also comments in (6, 7)].

Basic Facts to Consider in Applying the Concept of Xenosialitis to Humans

The main differences regarding xenosialitis between humans and CMAH-deficient mouse models in terms of prevailing conditions are as follows: (1) the actual levels of loading among human cells with diet-derived Neu5Gc, and (2) the differences between A-GcAbs that result either from immunization by diet or from *active* immunization with animal-derived products and adjuvants.

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Levels of Neu5Gc-Loading Among Human Cells With Diet-Derived Neu5Gc

The presence of Neu5Gc traces on ECs or epithelial cells from various organs in humans has been established using ten autopsy samples (8). Since unambiguously observing such deposits using anti-Neu5Gc chicken polyclonal Ab staining on frozen or fixed-histological tissue samples was difficult, we used flow cytometry to assess the binding of anti-Neu5Gc chicken Abs on living ECs from large arteries of brain-dead donors (9). Although we confirmed a faint signal on gated ECs in four samples, three other preparations were found to be negative (9). A roughly similar proportion was found in eight additional living EC preparations that were tested after sorting, of which, two were positive, two were negative, and four had extremely faint or negative staining (*unpublished data*). This is distinguished from the high-Neu5Gc loading among certain malignant cells (10), which may enable therapy using exogenously produced A-GcAbs and to monitor A-GcAb levels as a disease marker (11, 12). The low metabolic incorporation of Neu5Gc in humans with a Neu5Gc-rich diet can be explained by intestinal colonization of sialidases producing bacteria (13, 14).

Few studies have investigated Neu5Gc loading among human cells (8, 9); however, further studies are warranted to establish whether indeed a substantial fraction of humans actually lack the fundamental component of the theoretical basis of the xenosialitis model. Although Neu5Gc exists at trace levels on ECs of positive individuals, we must also consider the myriad of surface glycoproteins or lipoproteins that display Neu5Gc and the high diversity of Neu5Gc terminal residues, which result in a huge dispersion of cell-surface epitopes that are potentially recognized by A-GcAbs (15). It is thus possible that, following interactions with A-GcAbs, the coexistence of trace levels of antigens and the high epitope dispersion results in physiological cellular signaling that is below the activation threshold. In a recent analysis of affinity-purified natural A-GcAbs detected by ELISA and Glycan arrays, the authors suggested that "specific A-GcAbs" may only represent a small minority of the pool detected in the assays (16). These data confirm our working hypothesis. In addition, whatever the molecular definition of the glycans recognized by the natural, diet induced, anti-Neu5Gc measured by ELISA or Arrays, the question of their biological effects in humans remains.

Indeed, only a few *in vitro* studies explored the effects of A-GcAbs on human cells. The first (17) suggests there is an activation of umbilical ECs that develop a white blood cell binding phenotype after incubation with A-GcAbs-containing whole serum. However, these first experiments used several extra-physiological conditions; for instance, the Neu5Gc loading among ECs far exceeding the levels naturally observed in human ECs and the high anti-Neu5Gc titer of the serum tested. A second study (9) used affinity-purified A-GcAbs from either normal sera (diet-induced Abs) or sera of those highly immunized by rabbit polyclonal IgGs (elicited Abs) (18). In addition, large artery ECs that undergo physiological loading levels of Neu5Gc were used (9).

Although this last study (9) was restricted to the complete transcriptomic patterns and apoptosis of stimulated ECs, it is interesting that the activation patterns triggered either by purified diet-derived human A-GcAbs or by rabbit IgG-elicited A-GcAbs in these more physiological conditions did not present a classical "inflammation-like" activation of ECs. In contrast, the observed patterns are consistent with the concept that A-GcAbs may contribute to the homeostasis of ECs (9). Moreover, purified A-GcAbs were shown to downregulate classical inflammation patterns that are induced by the presence of normal sera, added as a complement source (with components also necessary to cell homeostasis) (9). Further, purified A-GcAbs inhibited important master genes involved in EC activation (9). In conclusion, the theoretical basis of xenosialitis in humans, which involves A-GcAbs, requires an improved assessment of the actual levels of Neu5Gc loading among human cells *in vivo* and of the percentage of normal individuals who exhibit detectable Neu5Gc on ECs or epithelia. In addition, the effects of purified A-GcAbs on ECs or epithelial cells should be tested *in vitro* under experimental conditions that more closely mimic "physiological" Neu5Gc loading.

Differences Between Anti-Neu5Gc Abs That Result From Immunization by Diet and Those Elicited by Active Immunization With Animal-Derived Products

Humans develop A-GcAbs within the first few months of life after being introduced to a Neu5Gc-containing diet (19). The impact of food antigens on immunity is poorly understood; further, the apoptosis of diet-activated T cells is a hallmark of the healthy intestine (20). Whether diet/microbiota levels significantly affect A-GcAb levels in healthy adults has not yet been determined (21). In contrast, after implantation of animal biodevices (22, 23) or infusion of animal-derived molecules, such as rabbit IgGs, blood-elicited A-GcAb levels drastically increase for several months (18) and largely exceed the average normal levels in non-immunosuppressed individuals. As expected, these "exogenously" elicited Abs display a high affinity and altered repertoire (24). In contrast to diet-derived natural immunization, the elicited responses result in a vigorous, memory-type induction of A-GcAbs in young adults (18) with a significant number of individuals exhibiting extremely high titres (from 20 μg/ml up to 1 g/l). The extent to which proportion-elicited A-GcAbs stemmed from B cells that were primed by diet-derived Neu5Gcs is currently unknown. Importantly, exposure to such high titres of A-GcAbs affects drug half-life and is associated with the serum sickness disease (SSD), likely due to the A-GcAbs (25). However, SSD is linked to immune complexes that circulate (26), rather than *in situ* xenosialitis. Whether the increase in late renal failure in those who develop SSD (25) results from early graft injury due to immune complexes, or xenosialitis that results in long-term exposure to elicited A-GcAbs, remains unknown. The late loss of transplant function that is associated with the highest elicited A-GcAb titres in patients who received rabbit IgGs [in Supplementary Data of (25)] is yet anecdotal,

Abbreviations: A-GcAbs, Anti-Neu5Gc antibodies; CAD, Coronary artery disease; CMAH, Cytidine monophosphate N-acetylneuraminic acid hydroxylase; CRC, Colorectal cancer; EBV, Epstein-Barr virus; IMN, Infectious mononucleosis; ECs, Endothelial cells; Neu5Gc, N-glycolylneuraminic acid; MS, Multiple sclerosis; SSD, Serum sickness disease.

TABLE 1 | Clinical correlations between died-induced A-GcAbs and pathologies.

Pathologies	Aim/rational	Clinical findings	Significance and limits	References
Malignancy	Investigate whether anti-Neu5Gc antibodies increase the risk of colorectal cancer (CRC).	No correlation was found between antibodies against Neu5Gc alone or against individual Neu5Gc-bearing epitopes and CRC. However, a sialoglycan microarray study demonstrated a positive association of CRC risk and total anti-Neu5Gc-glycan antibody responses.	Whilst a correlation of CRC with the total anti-Neu5Gc antibody response has been demonstrated, a link with causation is still lacking. High Neu5Gc loading of malignant cells may also boost anti-NeuGc levels.	(4)
	Investigate whether anti-Neu5Gc elicited by rabbit IgGs are associated with a higher incidence of colon carcinoma.	There was no evidence that exposure to high levels of elicited anti-Neu5Gc antibodies is associated with a higher incidence of colon carcinoma.	This study relies on indirect evidence: ATG-treated renal allograft recipients have high-level anti-Neu5Gc antibodies.	(28)
Vascular diseases	Investigate if the levels of A-GcAbs correlate with chronic vascular lesions.	No correlation of A-GcAbs with coronary artery disease (CAD).	Case-control study using three tests for A-GcAbs (835 CAD vs. 1869 controls).	(21)
	Investigate if the levels of A-GcAbs correlate with acute vascular lesions.	No acute vascular pathology reported in young type 1 diabetic patients with extremely high titers of elicited A-GcAbs.	Deals with *elicited* A-GcAbs.	(18, 27)
		Inverse correlation of A-GcAbs and arterial lesions in Kawasaki disease.	Case-control study. No reported vascular pathologies.	(29)
		Increase A-GcAbs in acute EBV primo infection (IMN).	No reported vascular lesion in IMN.	(30)
Infertility	A-GcAbs could block the capacitation and migration of Neu5Gc-loaded spermatozoid, and egg fecundation and implantation in the female uterine tract exhibiting Neu5Gc.	No correlation between the presence of Neu5Gc or A-GcAbs in uterine tract and semen quality or uterine pathology.	Only a minority of men, even from infertile couples, incorporated Neu5Gc in sperm. Interesting hypothesis but limited number of cases studied yet.	(31)
Asthma	Investigate whether exposure to Neu5Gc is involved in the protection against allergy, asthma, and inflammatory bowel disease observed in children exposed to farm environment.	Farmers' children had elevated levels of anti-Neu5Gc antibodies that were inversely correlated with wheezing and asthma in non-atopic subjects.	Significant limit: the authors speculate that Neu5Gc behaves *in vitro* as an anti-inflammatory molecule in humans. However, free circulating Neu5C is controversial *in vivo*.	(32)
Multiple sclerosis	Possible effect on Brain Blood Barrier permeability	A-GcAb reactivity towards some Neu5Gc-bearing synthetic glycans in MS patients.	No increase of A-GcAbs in the blood of MS patients in another study.	(30, 33)

generated by a small group of patients in the absence of graft histological samples. There are thus two different contexts that must be considered. Deleterious-elicited A-GcAbs (as tested experimentally in CMAH-deficient mice) do not imply that diet-induced "natural" A-GcAbs are necessarily detrimental. Since extremely high titres of elicited A-GcAbs in non-immunosuppressed patients were not associated with even a clinically detectable acute vascular insult (27), coevolution adaptation to diet-induced A-GcAbs may also operate to control elicited A-GcAb effects. Similarly, diet-induced A-GcAbs within the first year of life are not associated with vascular pathology. Thus, along with the "threshold effect" hypothesis, the presence of protective mechanisms, which are likely shaped by evolution to escape the deleterious effects of A-GcAbs, is another working hypothesis to consider.

A-GcAb Levels in Human Diseases—Particularly in Cases in Which Animal Models Suggested a Possible Role of Xenosialitis (Table 1)

Elevated A-GcAb levels have been determined to be inversely correlated with non-atopic asthma in farmers' children (32). Likewise, elevated A-GcAb titres have also been observed in patients with normal coronaries in Kawasaki disease (29). Both observations do not suggest a link between A-GcAb titres and inflammation.

Xenosialitis has been proposed as a contributor to colon cancer, due to also being associated with high red meat intake (21). However, no association between A-GcAb levels, red meat intake, or coronary artery disease (CAD) risk, has been evidenced in adults, as assessed by several types of ELISA (21). In comparison, colorectal cancer (CRC) was found to be significantly associated with total A-GcAb responses using a Sialoglycan Microarray that measures the Ab repertoire against Neu5Gc-glycans (21). Nevertheless, the increased A-GcAb titres observed may merely represent an immunogenic marker of the strong Neu5Gc loading of malignant cells (10, 12, 34). Another study compared the incidence of colon cancer between kidney recipients (including 212,465 patients and 522 with colon cancer) who either received or did not receive rabbit anti-T cell IgGs that are able to induce long-term elevated A-GcAbs (28). While relying indirectly on inducing elicited A-GcAbs by rabbit IgGs in immunosuppressed patients (25), a long-term survey showed no difference in colon cancer incidence (28).

In further studies, the deleterious effects of A-GcAbs from females (and potentially males) have been reported on spermatozoids and egg implantation in the female uterine tract that contain Neu5Gc from diet, which thus may imply that xenosialitis is involved in certain cases of infertility (31). However, neither the presence of Neu5Gc nor that of A-GcAbs have been correlated with differences in semen quality or the presence of uterine pathology (31).

There are also several instances in the clinical arena in which a low antigen density on ECs does not result in identifiable deleterious effects, such as when blood group A2 organs are transplanted in ABO-incompatible recipients (35). Moreover, normal individual sera display a high diversity of Abs that cross-react with self-determinants (36, 37), which may provide anti-apoptotic signals and shape the immune repertoire by enabling more efficient cognitive responses. Significantly increased levels of A-GcAbs have also been reported in EBV acute infectious mononucleosis (IMN), which is likely related to the concomitant high percentage of EBV-infected B cells (30). As IMN is associated with high incidence of multiple sclerosis (MS) (38), it has been hypothesized that A-GcAb levels could enhance the migration of anti-EBV T cells through the blood-brain barrier (39). However, as previously mentioned, transcriptomic studies *in vitro* do not reveal patterns that are classically associated with EC inflammation (9). Using a semi-quantitative synthetic glycan array, a recent study reported a specific pattern of IgG reactivity for some Neu5Gc epitopes in MS patients compared to other neurologic diseases (33).

Thus, whether the concomitant traces of Neu5Gc on ECs and of diet-induced circulating A-GcAbs theoretically trigger inflammation at the site of the antigens, either by direct or complement-mediated effects, or by bridging CD16 positive blood mononucleated cells onto ECs, remains to be explored. Due to the absence of convincing and statistically-powered clinical evidence of xenosialitis, we recommend critically revisiting associated concepts and exploring the possibility that diet-derived A-GcAbs may contribute to EC homeostasis.

CONCLUSION

As Galileo said, experiments are "questions asked to nature," and so scientists routinely encounter subjectivity in their designs. We are aware that this limitation also exists when elaborating on the putative role of diet-induced "natural" A-GcAbs in the clinical arena, especially following Descartes' "*de omnibus dubitandum*" seminal warning. We suggest that a revisiting of the role of A-GcAbs in human biology with new tools and innovative working hypotheses will benefit scientific understanding and clinical application.

AUTHOR CONTRIBUTIONS

All authors thoroughly discussed all assertions of the correspondence and wrote this opinion paper.

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