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1 **Ganglioside GM3-Based Anticancer Vaccines: Reviewing the Mechanism and**
2 **Current Strategies**

3 (Running title: Review of GM3-Based Anticancer Vaccines)

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1 **Abstract**

2 Ganglioside GM3 is one of the most common membrane-bound glycosphingolipids. The
3 over-expression of GM3 on tumor cells makes it defined as a tumor-associated carbohydrate
4 antigen (TACA). The specific expression property in cancers, especially in melanoma, make it
5 become an important target to develop anticancer vaccines or immunotherapies. However, in
6 the manner akin to most TACAs, GM3 is an autoantigen facing with problems of low
7 immunogenicity and easily inducing immunotolerance, which means itself only cannot elicit a
8 powerful enough immune response to prevent or treat cancer. With a comparative
9 understanding of the mechanisms that how immune system responses to the carbohydrate
10 vaccines, this review summarizes the studies on the recent efforts to development GM3-based
11 anticancer vaccines.

12
13 **Keywords:** GM3, TACA, Anticancer Vaccine, Glycoconjugate, Glycoimmunology

14
15 **Abbreviations:**

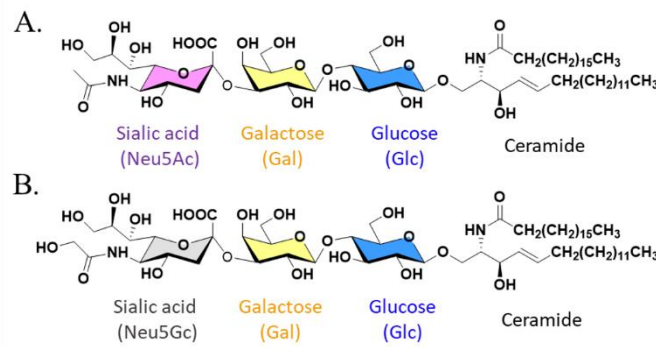
16 **APC**, antigen-presenting cell; **BCR**, B cell receptor; **BSA**, bovine serum albumin; **CDC**,
17 complement dependent cytotoxicity; **CMAH**, cytidine mono-phospho-*N*-acetylneuraminic acid
18 hydroxylase; **COSMC**, core1-specific molecular chaperone; **DC**, dendritic cell; **ELISA**,
19 enzyme-linked immunosorbent assay; **TACA**, tumor-associated carbohydrate antigen; **GSL**,
20 glycosphingolipid; **HSA**, human serum albumin; **iNKT**, invariant natural killer T; **KLH**,
21 keyhole limpet hemocyanin; **LacCer**, lactosylceramide; **LPS**, lipopolysaccharide; **mAb**,
22 monoclonal antibody; **MHC**, major histocompatibility complex; **MPLA**, mono-phosphoryl
23 lipid A; **OVA**, ovalbumin; **PAMP**, pathogen-associated molecular pattern; **PRR**, pattern
24 recognition receptor; **TCR**, T cell receptor; **TME**, tumor microenvironment; **TTox**, tetanus
25 toxoid; **TLRs**, toll-like receptors; **VSSP**, very small size proteoliposomes; **VLP**, virus-like
26 particle;

27 For monosaccharides: **Glc**, glucose; **Man**, mannose; **Gal**, galactose; **GlcNAc**, *N*-acetyl-
28 glucosamine; **GalNAc**, *N*-acetyl-galactosamine; **Fuc**, fucose; **Neu5Ac**, *N*-acetylneuraminic
29 acid; **Neu5Gc**, *N*-glycolylneuraminic acid

30

1 **1. Introduction**

2 Ganglioside GM3 is one of the most common membrane-bound glycosphingolipids (GSLs)
3 in tissues, which is composed of a sialic acid-containing tri-saccharide glycan attached to a
4 lipophilic ceramide (Figure 1A). This structure allows the GM3 to be anchored into the
5 phospholipid bilayer of eukaryotic cells with the ceramide moiety, leaving the glycan exposed
6 on the cell surface and performing a series of complex biological and physiological functions.
7 For example, GM3 rafting on cell membrane could form complexes with various co-localized
8 structures (e.g. growth factors, insulin receptors and tyrosine kinases) by non-covalent
9 (hydrophobic, electrostatic) bonds [1]. As a result, these complexes dynamically interact with
10 the glycolipid-enriched microdomains to regulate downstream switches, thereby regulating the
11 cellular signal transduction, cell proliferation, membrane transportation, and cell adhesion, etc
12 [2-4]. In addition to GM3 that exists in a lipid raft model on the cell surface, there is also a
13 small amount of free GM3 in the sera (6 µg/mL), and this content remains stable unless
14 metabolic-related diseases such as type 2 diabetes and hyperlipidemia causes a significant
15 increase (1.5-fold) in serum GM3 [5].



16 **Figure 1.** Representative structures of ganglioside GM3. **A.** Neu5Ac-GM3; **B.** Neu5Gc-GM3

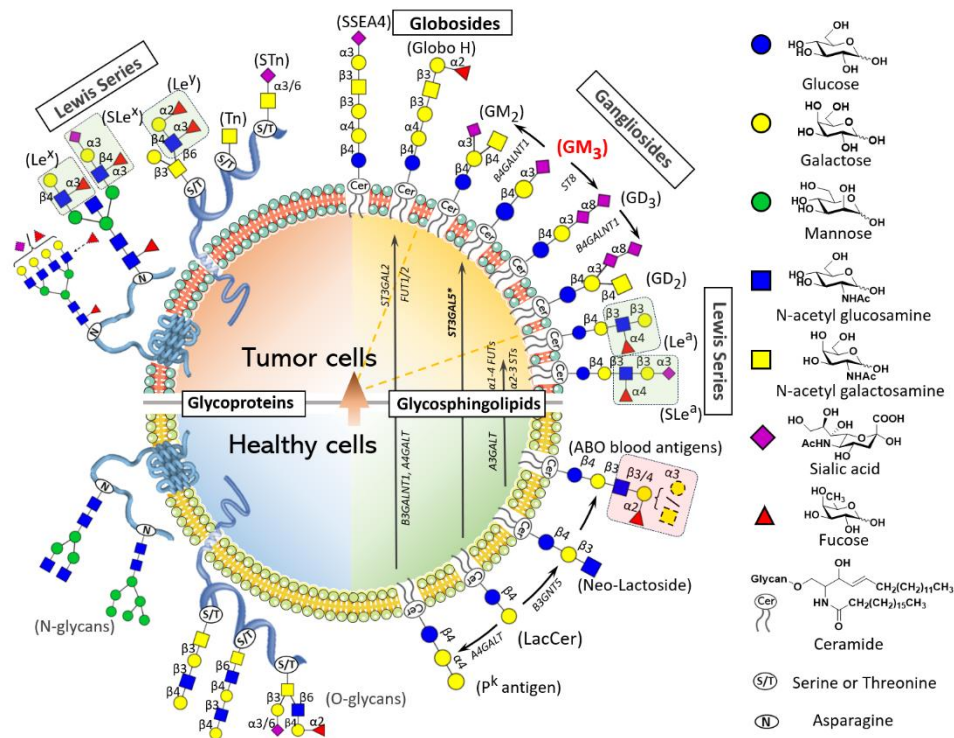
17 Ganglioside GM3 is over-expressed on some tumor cells, which makes it defined as a tumor-
18 associated carbohydrate antigen (TACA). Studies have already identified that GM3 is
19 significantly overexpressed in melanoma and not found in healthy skin or skin neighboring
20 primary melanoma [6, 7], and the content of GM3 on cancer cells is positively correlated with
21 the metastasis ability [8, 9]. It is worth mentioning that an important GM3 variant, Neu5Gc-
22 GM3 (Figure 1B), was detected in melanoma, colon and breast cancer cells and was not present
23 in other human healthy somatic cells [10, 11].

1 Since TACAs can play a role of antigenic epitopes and then trigger downstream immune
2 responses, immunotherapy research that aims to target GM3 has always been a research hotspot.
3 However, a major drawback of GM3 anticancer vaccines is the poor immunogenicity (ability
4 to induce immune response) and antigenicity (ability to bind immunoglobulins), due to the
5 autoantigen property which leads to immune tolerance and immune evasion [12, 13]. In
6 particular, the tumor microenvironment (TME) caused by the increase of sialylation during
7 tumor development will act like a "cloak" to protect tumor cells and reduce immune responses
8 [14]. With a comparative understanding of the mechanisms that how immune system responses
9 to the carbohydrate vaccines, this review summarizes the studies on the development of GM3
10 based anticancer vaccines in recent years.

11 **2. Glycosylation and tumor-associated carbohydrate antigens (TACAs)**

12 Glycosylation is an important post-translational pathway which enzymatically attaches
13 oligosaccharides to proteins and lipids to form glycoproteins and GSLs [15, 16]. Major
14 classification of glycoproteins in eukaryotic cells can be classified into *N*-glycosylated and *O*-
15 glycosylated glycoproteins [17]. As shown in Figure 2, the *N*-glycans sharing a penta-
16 saccharide core structure ((Man)₃-(GlcNAc)₂-), while the most common *O*-glycosylation is
17 starting from *N*-acetyl-galactosamine (GalNAc). GSLs consist of a hydrophobic ceramide (Cer)
18 moiety anchoring the outer layer of cell membranes and a glycan moiety extending into the
19 extracellular matrix due to the hydrophilicity [18]. Generally, the glycoside moiety of GSLs
20 starts from glucose, and various monosaccharides extend and branch the glycan structure under
21 the action of glycosyltransferases to form diverse complex oligosaccharides (e.g. p^k series, Neo-
22 Lactosides, Globosides and Gangliosides) [19]. The most representative human functional
23 GSLs are the ABO blood group antigen system. These carbohydrate antigens, present on red
24 blood cells, differ by a monosaccharide residue at the terminal end of the glycan, yet they
25 impose significant limitations on blood transfusion compatibility [20]. There is new evidence
26 that mammals use RNA as a third scaffold for glycosylation. Those so-called "glycoRNAs"
27 which consisted of small noncoding RNAs and sialylated glycans were present in multiple cell
28 types and can interact with anti-dsRNA antibodies and members of Siglec receptors [21]. This
29 discovery will greatly promote research in the fields of glycoscience and help scholars more

1 clearly understand the important role of glycans in life activities.



2 **Figure 2.** Overview of the representative human cell glycosylation and tumor associated carbohydrate antigens
 3 (TACAs). *N*-glycans possess a common structure of (Man)₃(GlcNAc)₂ and are linked to the asparagine side
 4 chains on membrane proteins; most *O*-glycans start with GalNAc and are linked to serine or threonine residues
 5 on proteins; glycosphingolipids are anchored to the cell membrane via their hydrophobic lipid tails. Under the
 6 action of glycosyltransferases and glycosidases (some examples marked on the black arrows), a wide variety
 7 of glycans are elongated and branched, performing biological functions. During cancer initiation and
 8 progression, abnormal glycosylation leads to the generation of various specific TACA structures (e.g. SSEA4,
 9 Globo H, GM2, GM3, GD3, GD2 and Lewis Series. Of them, GM3 is highlighted in red). To explain, there are
 10 only some representative glycans to illustrate the abnormal glycosylation. On cancer cells, abnormal
 11 glycosylation manifests as an increased or decreased trend in glycan alterations, which means the various
 12 structures presented here are not exclusively expressed in either normal or cancer cells.

13 During the development of cancer, aberrant glycosylation may occur in both glycoproteins
 14 and glycolipids. For the *N*-glycans, based on the common penta-saccharide core structure, the
 15 tumor-related aberrant glycosylation often manifests as multi-antennary galactose branching
 16 [22], bisecting GlcNAc branching [23], fucosylation and sialylation [24, 25]. The *O*-glycans
 17 normally form as extending and branching oligosaccharides beginning with GalNAc. However,
 18 in cancers, because of the mutations in the core1-specific molecular chaperone (COSMC) and
 19 competition of the sialyltransferase ST6GalNAc-I, it always shows an over-expression of the
 20 Tn (“Tn” stands for truncated) antigens or sialyl Tn (STn) in cancer related glycoproteins [26].
 21 One of the most thoroughly studied breast cancer associated glycoprotein is mucin, which is a

1 type I transmembrane glycoprotein with a unique extracellular domain consisting of lots of
2 tandem repeat of 20 amino acids (HGVTSAPDTRPAPGSTAPPA) providing abundant Ser and
3 Thr as potential sites for Tn or STn *O*-glycosylation [27].

4 As the most basic precursor of GSLs, lactosylceramide (LacCer) leads to the ganglioside-
5 series pathway. GM3 is synthesized by the addition of a sialic acid to LacCer with a CMP-sialic
6 acid donor catalyzed by the sialyltransferase ST3GAL5. Further extension by another
7 sialyltransferase ST8 allows CMP-sialic acid to attach onto the position-8 of sialic acid and
8 then form GD3 and other poly-sialylated gangliosides. Under the catalysis of glycotransferase
9 B4GALNT1, GM3 and GD3 can extend the GalNAc branch to produce the corresponding
10 ganglioside GM2 and GD2 [28]. In the similar way, other types of GSLs are extended into
11 globoside TACAs such as SSEA4 and Globo H under the action of abnormally overexpressed
12 glycosyltransferases in tumors, or produce special terminal structures such as the Lewis series.
13 For more than twenty years, these TACAs mentioned above have demonstrated potential
14 usefulness in strategies for cancer immunotherapy [29]. Some of them have already been well-
15 developed: e.g. Racotumomab[®] (anti-Neu5Gc-GM3 monoclonal antibody (mAb) for non-small
16 cell lung carcinoma), Theratope[®] (STn-keyhole limpet hemocyanin (KLH) glycoconjugate
17 vaccine for breast cancer) and OPT-822 (Globo H-KLH glycoconjugate vaccine with adjuvant
18 OPT-821 for breast cancer) have been achieved phase III clinical trials [30-32]. The approval
19 of Dinutuximab[®] (a chimeric anti-GD2 mAb) for the treatment of high-risk neuroblastoma
20 represents a significant achievement, marking the first endorsement by the U.S. Food and Drug
21 Administration (FDA) for a novel agent targeting a TACA in 2015 [33].

22 In short, glycosylation is an extremely complex, delicate and systematic molecular regulation
23 process. It gives tumors special cell epitopes, special signal transduction pathways and special
24 TME. All of these provide unlimited possibilities for developing immunotherapy targeting
25 TACAs, but also pose great challenges to researchers.

26 **3. Expression of ganglioside GM3 on cancer cells**

27 Ganglioside GM3 is widely present in virtually all types of animal cells and exhibits over-
28 expression in various human cancers, including melanomas, lung, and brain cancers [34, 35].
29 GM3 expression in melanomas has been extensively investigated. Reports indicate a

1 significantly higher percentage of GM3 existence in both primary and metastatic melanomas
2 compared to GD2 [36]. Additionally, there is a strong correlation between GM3 and GD3
3 expressions in both primary and metastatic melanomas. Notably, GM3 is absent in normal skin
4 and the skin adjacent to primary melanomas, in contrast to GD3. The onset of GM3 expression
5 occurs at an early stage of melanocyte differentiation, facilitating entry into the dermis of the
6 skin [7, 37]. The detection of GM3 on the surface of tumor cells is a critical determinant of the
7 metastatic phenotype [38]. Cell line B16 is derived from the classic melanoma mouse model
8 C57BL/6, which is well established and widely used as tumor model to study melanoma
9 metastases, melanoma-specific immune cells reactions and pharmaceutical activities [39].
10 Studies have indicated that GM3 density can influence antigenicity. The anti-GM3 mAb M2590
11 interacts with GM3 over-expressed melanoma B16 cells while exhibiting no reactivity with
12 corresponding normal tissue [40]. Therefore, researchers generally believe that the densely
13 packed cluster formed by GM3 on the melanoma cell membranes is the key to the action of
14 drugs or antibodies [41-43].

15 Considerable interest has been directed towards a GM3 variant, Neu5Gc-GM3, which is
16 distinguished by the presence of *N*-glycolylneuraminic acid (Neu5Gc) rather than *N*-
17 acetylneuraminic acid (Neu5Ac) [44, 45]. While Neu5Gc-GM3 is commonly synthesized in
18 most mammals, normal human cells lack this variant due to an inactivating mutation in the
19 human cytidine mono-phospho-*N*-acetylneuraminic acid hydroxylase (CMAH) gene [46].
20 However, Neu5Gc-GM3 is frequently observed at elevated levels in human cancers, including
21 melanoma, breast, colon and lung cancer [47, 48]. This phenomenon is attributed to the cancer
22 cell's metabolic incorporation of Neu5Gc derived from dietary sources, particularly red meat
23 [11]. The higher metabolic rate of Neu5Gc may result in the preference of expression by tumor
24 cells [49]. In a recent investigation, it was proposed that the succinate dehydrogenase complex's
25 subunit B, possessing a Fe₂S₂ domain resembling the deleted gene fragment of the human
26 CMAH protein, might interact with this protein, offering an alternative pathway for generating
27 Neu5Gc during hypoxia [50]. This may offer an explanation why metabolically active tumor
28 cells exhibit such an abnormal expression of Neu5Gc-GM3.

29 Another GM3 associated molecule, GM3 lactone, was also found in human and mouse
30 melanoma cells [43]. The carboxyl group on sialic acid undergoes condensation with the 2-

1 hydroxyl group of the adjacent galactose, forming a lactone. This formation is likely facilitated
2 by the lower pH of TME within tumor cells and potentially influenced by a distinct
3 conformation of GM3 ganglioside induced on the tumor cell surface due to its local high density
4 [51]. The specificity of GM3 expression on tumor cells and the variant potential of the molecule
5 itself make them promising for the development of anti-tumor immunotherapy [52].

6 **4. Principles of glycoimmunology**

7 The development of vaccines against carbohydrates was first pursued to immunize against
8 bacterial pathogens. The surface of bacteria contains abundant lipopolysaccharide (LPS)
9 structures, of which the polysaccharides are generally rich in galactose and sialic acid [53, 54].
10 Mammals have an innate immune system with conserved pattern recognition receptors (PRRs)
11 that can recognize the certain glycopatterns from LPSs. For example, there are three major
12 classes of carbohydrate-recognized lectins as pathogen-associated molecular patterns (PAMPs),
13 galectins, Siglecs and C-type lectin receptors [55]. This is an early defense mechanism against
14 bacterial infections. In addition to this early protection, immunizing the body with a certain
15 dose of LPS antigen can activate a more specific humoral immune response, produce
16 corresponding immunoglobulin antibodies, and trigger a downstream immune cascade. It is
17 first time to report in 1917 that there was a specific soluble substance which could induce
18 antibodies for *Pneumococci*, and it was subsequently identified as polysaccharide [56, 57].
19 Nowadays, vaccines based on bacterial LPSs have been well developed and routinely used in
20 an immunization schedule to prevent people from *Haemophilus influenzae* type B,
21 *Streptococcus pneumoniae* and *Neisseria meningitidis* [58, 59].

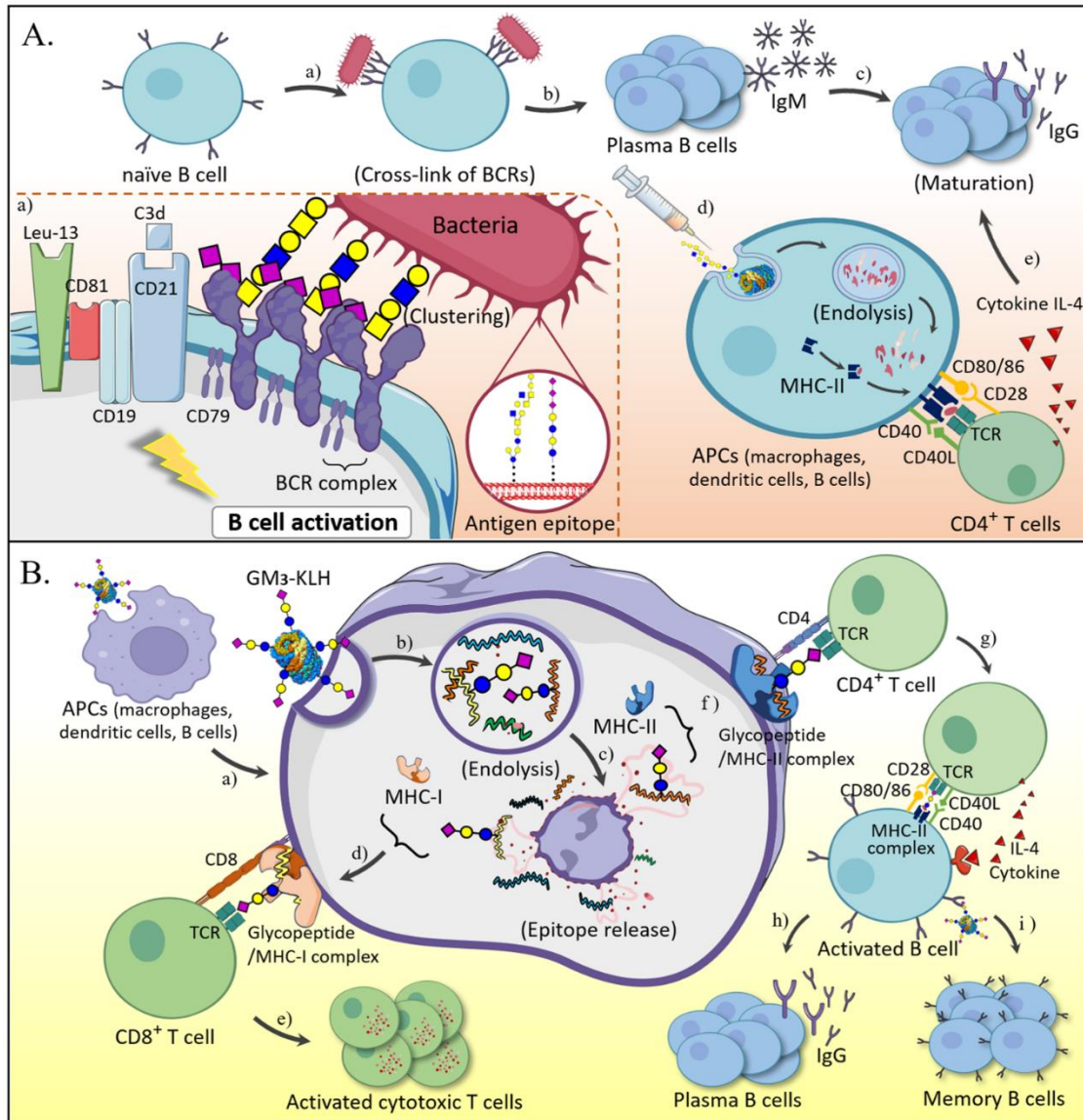
22 The application of bacterial LPS vaccines has confirmed that the effectiveness of glycans or
23 glycopatterns are immunogenic substances. Abnormal glycosylation and their corresponding
24 product TACAs become hallmarks of cancer and can serve as a source of novel clinical
25 biomarker developments, providing a set of specific targets for therapeutic intervention. Over
26 the past 30 years, the TACAs have demonstrated potential usefulness in strategies for cancer
27 immunotherapy, including the production of vaccines, mAb therapy and the generation of CAR-
28 T cells [60]. Of them, there are nine TACAs (GD2, GD3, GM3, Fucosyl-GM1, Globo H, poly-
29 sialic acid, Tn, STn, SLe^a) were explicitly included in the tumor-associated antigen list by the

1 National Cancer Institute of the United States [61]. Here, the three major immune pathways of
2 carbohydrate vaccines are compared.

3 **4.1 Polysaccharide vaccines activate B cells by crosslink to the receptor clusters**

4 Polysaccharide vaccines contain numerous identical antigenic epitopes and can stimulate B
5 cells through the cross-link interaction with the B cell receptors (BCRs) in a polyvalent fashion
6 [62]. This kind of humoral immune process is usually associated with the development of
7 antibacterial vaccines. In vaccinology it is widely used that inactivated pathogenic bacteria
8 (such as Bacillus Calmette-Guérin) or their polysaccharides isolated by bacterial lysis or
9 precisely synthesized by chemical methods [63, 64].

10 These polysaccharides will be firstly transported to the marginal zone of the spleen where
11 contains abundant naive B cells and enriched with the complement receptor 2 (also known as
12 CD21). The sketch of this pathway is shown in Figure 3A and described as follows: a) Bacterial
13 polysaccharides generally consist of many repeating glycopattern units with an average size of
14 40-80 nm [65]. When the B cells recognize and bind to such polymeric immunogens, the BCRs
15 (a complex consisting of immunoglobulin and CD79) spatially aggregate on the membrane. At
16 this point, BCRs will cluster into groups (10-20 BCRs per cluster), inducing B cell activation
17 and lowering the threshold for antigen affinity [66]. Clustered BCRs, with the co-activation of
18 complex of complement C3d and CD21, form a complex with CD19, CD81 and Leu-13,
19 activating downstream protein tyrosine kinases to activate B cells [67]. b) Then, the activated
20 B cells rapidly differentiate and proliferate into mature plasma B cells, producing IgM. The
21 production of IgM is a rapid process, typically occurring within the first week of primary
22 immunization. c) Upon successful initiation of the immune response to polysaccharides by this
23 pathway, B cells require further maturation in the germinal center of the spleen or lymph nodes:
24 transitioning from the IgM to the IgG isotype and differentiate to memory B cells which ensures
25 a more effective and long-lasting humoral immune protection [68]. Furthermore, due to the low
26 or absent expression of CD21 on B cells in children under 2 years of age, these polysaccharide
27 vaccines often fail to confer early immunity in newborns [69].



1 **Figure 3.** Schematic diagram of immune responses to vaccines with carbohydrate as antigen. **A.** T cell
 2 independent immune pathway triggered by bacterial lipopolysaccharides (LPS), and an example using LPS-KLH
 3 vaccine to demonstrate how glycoconjugates activate a T cell-dependent immune response: (a) To combine with the
 4 LPS exposed on the bacterial surface, the BCRs are spatially aggregated. Then the B cell intracellular signaling
 5 cascade is activated; (b) The activated B cells differentiate and proliferate into mature plasma B cells, producing
 6 IgM; (c) B cells maturation and switch to IgG production; (d) Immunized with LPS-KLH conjugate vaccine, the
 7 antigen presenting cells (APCs) uptake the glycoconjugates. Endolysis causes the decomposition of glycoconjugates
 8 into peptides or glycopeptides, and the glycopeptides are captured by the type-2 major histocompatibility complex
 9 (MHC-II), and transferred to the surface recruiting CD4⁺ helper T cells. (e) Activated helper T cells release cytokine
 10 IL-4 which further boost the maturation of B cells and induce the IgG production. **B.** T cell dependent immune
 11 pathway triggered by immunogenic glycoconjugate (GM3-KLH as example): (a) Glycoconjugate is endocytosed by
 12 APCs; (b) Endolysis causes the peptides and glycopeptides; (c) The epitopes are released and face different fates
 13 depending on peptide length; (d) Shorter peptide (8-10 residues) is captured by MHC-I and transferred to the surface
 14 recruiting CD8⁺ T cells; (e) Recognizing the presented GM3 antigen with the T cell receptor (TCR), cytotoxic T
 15 cells are activated and matured; (f) Longer peptide (10-25 residues) is captured by MHC-II and transferred to the
 16 surface recruiting CD4⁺ T cells; (g) The activated CD4⁺ helper T cell recognize the B cell which endocytosed and

1 presented the GM3 antigen. With activation of co-stimulatory molecules (CD28 and CD40L) by the corresponding
2 ligation, the helper T cell releases cytokines to boost the activation of B cell; (h) The activated B cells further
3 differentiate to plasma B cells and produce IgG, (i) or differentiate to memory B cells.

4 Carbohydrates are *per se* T cell-independent type 2 antigens which means the carbohydrate
5 itself only cannot activate the major histocompatibility complex (MHC) molecules involving
6 cellular immune pathway. To break this limitation and take advantage of the helper T cells,
7 glycoconjugate vaccines have been developed through the conjugation of polysaccharides to
8 highly immunogenic carrier proteins. The concept of glycoconjugate vaccines was first
9 developed by Avery and Goebel in 1931, of where they reported that the immunogenicity of
10 bacterial polysaccharides could be enhanced by covalently linking them onto an appropriate
11 carrier protein [70]. As illustrated in the supplementary pathway shown in Figure 3A: d) When
12 immunization with glycoconjugates containing specific carbohydrate antigen epitope from
13 those LPSs, the conjugates are internalized inside the antigen-presenting cells (APCs), e.g. B
14 cells, macrophages and dendritic cells (DCs). The protein component of glycoconjugates is
15 digested by proteases in the endosomal compartment, forming peptides and glycopeptides in a
16 slightly acidic environment within the endosomes. Some suitable fragments will bind within
17 the groove structure of MHC-II molecules, forming a peptide/glycopeptide-MHC-II complex.
18 This complex will then be transported to the surface of B cells, recruiting CD4⁺ T cells through
19 CD40 and CD80/86. When the αβ T cell receptor recognizes the glycopeptide-MHC-II complex
20 and co-stimulated by the ligand-binding of CD40L and CD28, the CD4⁺ T cells release
21 interleukin-4 (IL-4). e) Interaction with IL-4 makes the B cells to induce IgM to IgG class
22 switching (B cell maturation) that results in polysaccharide-specific high-affinity IgG antibody
23 production [71]. This T cell dependent pathway also allows the differentiation of antigen-
24 specific memory B cells and T cells and provides efficient prophylactic immunity [72].

25 **4.2 Glycoconjugate vaccines activate the helper T cells by MHC presenting**

26 Given that glycans can be recognized by the immune system and immunoglobulins, targeting
27 TACAs on tumor cells represents a promising avenue for the development of cancer
28 immunotherapy. Compared to the bacterial polysaccharide antigens, TACAs have a lower
29 degree of polymerization, consisting of oligosaccharides composed of 2~8 monosaccharide
30 units [73, 74]. They cannot directly trigger the B cell immune pathway because the size of

1 TACA molecules is not enough to cross-link to BCRs and make the BCRs come into cluster
2 [66]. After it was proven that glycoconjugates with immunogenic carrier proteins can
3 effectively activate the MHC dependent T cell pathway, making TACA molecules in form of
4 glycoconjugates made it possible to develop carbohydrate based anti-tumor vaccines [75].

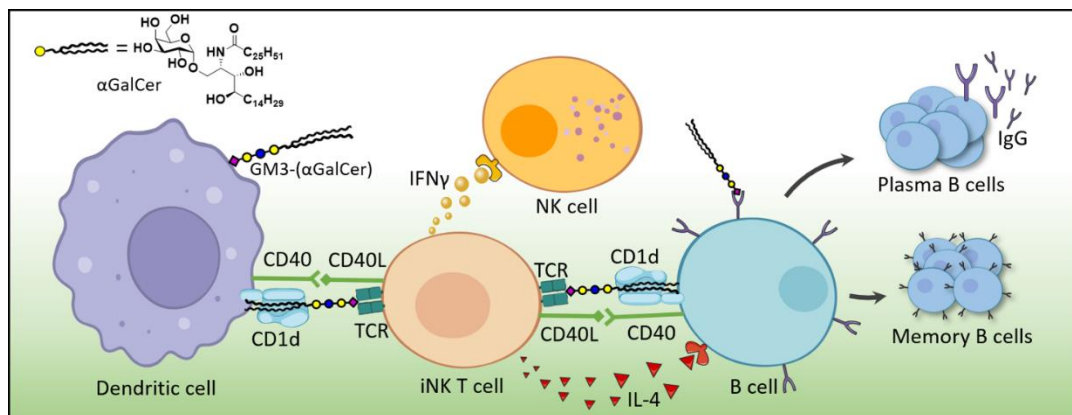
5 The strategy to utilize TACA based glycoconjugate vaccines is based on the classic adaptive
6 immune system, also known as the acquired immune system, which relies on the ability to
7 distinguish “self” antigens from pathogen-derived antigens [76]. Take the immune process of
8 GM3-KLH glycoconjugate as an example, this pathway is illustrated as Figure 3B: a) Upon
9 conjugation an immunogenic carrier protein, the glycoconjugates will be firstly recognized as
10 foreign substances and swallowed by APCs, including macrophages, DCs and B cells [77]. b)
11 During this endocytosis process, the glycoconjugate molecules are surrounded by an area of
12 cell membrane, which then buds off inside the APCs to form a vesicle containing the ingested
13 contents. c) After internalization into APCs, the glycoconjugates are digested by proteases to
14 release peptides, and glycopeptides bringing TACAs. d) Specific 8-10 residue long peptides or
15 glycopeptides are then transported to the lumen of endoplasmic reticulum for loading on MHC-
16 I to form a complex [78]. In particular, there are two long α -helices (45 kDa of each) form two
17 walls of a groove-shape like structure. The proper peptides could lie into the groove, and, in the
18 case of glycopeptides, the glycans will extend out [79, 80]. The glycopeptide-MHC-I complex
19 are then transferred onto the cell surface of APCs and recruit $CD8^+$ T cells. e) The T cell receptor
20 (TCR) is a disulfide-linked membrane-anchored heterodimeric protein normally consisting of
21 the highly variable α - and β -chains known as $\alpha\beta$ -TCR (95% of all TCRs). The remaining 5% is
22 $\gamma\delta$ -TCR composed of γ - and δ -chain [81]. Studies have shown that $\gamma\delta$ -TCR is related to the
23 recognition of the glycan moiety presented by the glycopeptide-MHC-I complex [82]. Specific
24 TCR interacting with the antigen presented by MHC-I leads to activation and proliferation of
25 the T cells, which further differentiate to mature cytotoxic T cells [83]. These cytotoxic cells
26 will act like the “monitors” to monitor tissues and cells according to the specific antigen
27 information received, e.g. GM3 as example. If cancer cells expose the GM3, they will be
28 eliminated by these cytotoxic T cell by downstream pathways.

29 On the other hand, these glycopeptide fragments also activate B cells and produce antibodies
30 under the $CD4^+$ T cell assisted pathway: f) In this pathway, glycopeptides bind to MHC-II

1 molecules to form a complex. Similarly, MHC-II consists of an α (30-34 kDa) and a β (26-
 2 29kDa) subunit to form a groove-like structure with openings at both ends which allows a
 3 longer peptide (10-25 residue long) could be accommodated [84, 85]. Export of the
 4 glycopeptide-MHC-II complex to the surface of APCs allows interactions with TCRs on CD4⁺
 5 T cells to initiate effector and regulatory functions. g) In particular, the activated CD4⁺ T cells
 6 (also known as helper T cells) can make similar interaction with B cells. h) Thereby, the
 7 activated B cells are instructed by interleukins to mature and produce IgG antibodies. i) The
 8 memory B cells are also differentiated in this step.

9 4.3 Glycolipid vaccines activate the iNKT cells by CD1d presenting

10 In recent years, a strategy has emerged that uses glycolipid molecules as immune agonists to
 11 present oligosaccharide antigens and generate immune responses. The glycolipids are found to
 12 be immunomodulatory molecules which have the ability to activate a unique subset of T
 13 lymphocytes, known as invariant natural killer T (iNKT) cells [86]. Glycolipid antigens
 14 (example: α -galactosylceramide, α GalCer) are recognized by iNKT cells with the presentation
 15 by CD1d, an MHC-I-like molecule (Figure 4). The CD1d molecule is nonpolymorphic,
 16 therefore, it allows the certain ligand α GalCer to fit the lipid chain into cavity of CD1d and
 17 leave the TACA moiety outside to be recognized by TCR on iNKT cell [87]. After being
 18 activated, iNKT cells rapidly release a diverse array of Th1 cytokines (e.g. interferon- γ , IFN γ)
 19 and Th2 cytokines (e.g. IL-4) to modulate natural killer (NK) cells and B cells responses,
 20 respectively [88, 89]. Besides, the generated cytokines could also induce activation cascades of
 21 other immunocompetent cells, such as CD4⁺ T cells, CD8⁺ T cells, DC cells. Thus, α GalCer
 22 has been called a “universal helper” due to its powerful self-adjuvant properties [35].



23 **Figure 4.** Pathway of immunomodulation by invariant natural killer T (iNKT) cells. The dendritic cells (DCs) present

1 the antigen GM3 by the α GalCer receptor CD1d on DCs (structure of α GalCer shown in the top left). After the TCR
2 ligation, the iNKT cells are activated and then secrete multiple Th1- and Th2-type cytokines, such as IFN γ and IL-
3 4, influencing the activation of natural killer (NK) cells and B cells, respectively.

4 **5. Strategies to boost immune response of GM3 vaccines**

5 Because of immunological tolerance towards tumor antigens, the immunogenicity of self-
6 antigens, particularly small carbohydrates and glycopeptides in synthetic vaccines, is
7 insufficient to provoke a robust immune response. Hence, besides the specificity, vaccines must
8 possess sufficient potency to overcome tolerance and reestablish surveillance. The most direct
9 and efficient approach is to couple GM3 to highly immunogenic carriers. Another method to
10 break immune tolerance to GM3 involves employing modifications onto GM3 or structural
11 analogs. These GM3 derivatives could potentially exhibit heightened immunogenicity and
12 provoke a vigorous antibody production which may have cross-reactivity with native GM3.
13 Here, an introduction will be provided from both the aspects of the varied immunogenic carriers
14 and GM3 derivatives.

15 **5.1 Exploit more effective carriers**

16 In the context of sugar-based vaccine carriers, the primary consideration revolves around the
17 immunogenicity of the carrier itself, including the antigenic epitope sequences of heterologous
18 protein carriers, or the patterned ligands recognized by the human humoral immune system of
19 the non-protein carriers. In addition to this, the size effect is also taken into account in vaccine
20 delivery. Studies have demonstrated that vaccines with larger size exhibit enhanced interactions
21 with APCs, such as those present on whole cells, virus-like particles, and liposomal delivery
22 systems [90].

23 **5.1.1 Protein carriers**

24 Covalently conjugating TACAs with immunogenic proteins has been successfully applied to
25 develop anticancer vaccines. Over the years, carrier proteins like KLH, tetanus toxoid (TTox),
26 cross-reactive material 197 (CRM197), bovine serum albumin (BSA), human serum albumin
27 (HSA) and ovalbumin (OVA) have been extensively utilized in glycoconjugate vaccines (Table
28 1). The covalent conjugation of TACAs with these carriers has proven to significantly enhance
29 the immune response [91-93].

Table 1. Common Immunogenic Protein Carriers for TACAs

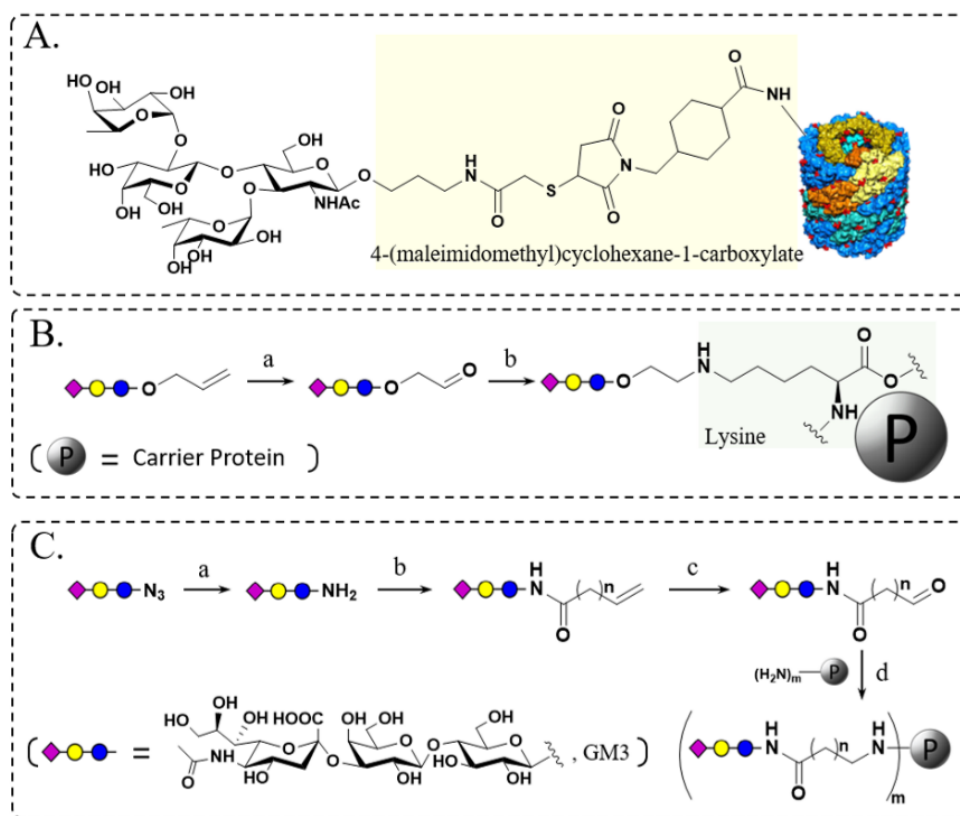
	Molecular Weight	Glycosylation	Multimerization	Amino acid count.	Lysine composition	Database entry
KLH (Keyhole limpet Hemocyanin)	392.2 kDa (S-1) ^a	6 <i>N</i> -sites ^b	decamers/ didecamers of S-1/2	3414	293 (8.58%)	PDB: 4BED UniProt: Q10583
	391.5 kDa (S-2)	3 <i>N</i> -sites		3421	288 (8.42%)	Q10584
CRM197 (Detoxified diphtheria toxin)	61.6 kDa	no	no	567	41 (7.23%)	PDB: 4AE0 UniProt: P00588
TTox (Tetanus toxoid)	150.7 kDa	no	no	1315	114 (8.67%)	PDB: 5N0C UniProt: P04958
HSA (Human serum albumin)	69.4 kDa	23 <i>N</i> -sites	no	609	64 (10.51%)	PDB: 1AO6 UniProt: P02768
BSA (Bovine serum albumin)	69.3 kDa	1 <i>N</i> -sites	no	607	65 (10.71%)	PDB: 3V03 UniProt: P02769
OVA (ovalbumin)	42.9 kDa	1 <i>N</i> -sites	no	386	32 (8.29%)	PDB: 1JTI UniProt: P01012

2 ^a (S-1/2): two subunits of KLH sharing around 60% identity at the protein level;

3 ^b *N*-site: number of identified *N*-glycosylation sites provided by the database UniProt (<https://www.uniprot.org/>).

4 In order to make appropriate sugar-protein coupling, the linker holds significant importance.
5 They increase the efficiency of conjugations by lessening the steric hindrance. Besides, the
6 proper linkers provide more flexible rotation and enhance the exposure of antigenic epitopes
7 on the surface of candidates [94]. A number of methods, such as reductive amination,
8 photocatalyzed thiol-ene reaction, azide-alkyne Click reactions and others, have been devised
9 to facilitate the glycoconjugation of selected molecular and biomolecular entities [95]. However,
10 some structurally complicated linkers may induce the production of anti-linker antibodies,
11 which can suppress the IgG antibody response against TACAs. Boons and colleagues found
12 that a Lewis^y antigen conjugated to KLH using a rigid cyclic 4-(maleimidomethyl)
13 cyclohexane-1-carboxylate linker suppressed the IgG antibody response against the Lewis^y
14 epitope (Figure 5A). A thorough analysis of sera through enzyme-linked immunosorbent assay
15 (ELISA) confirmed a robust IgG antibody response going against the linker region. Therefore,
16 selecting a structurally simple and flexible linker can avoid this problem. A common strategy
17 is to introduce an ethylenic bond onto the reducing end glycosidic linkage of oligosaccharides,
18 followed by oxidation to form an aldehyde [96]. Subsequently, the oligosaccharide is coupled
19 to the lysine side chain of the carrier protein through a reductive amination reaction (Figure
20 5B), and it has been widely applied to the conjugation of GM3. Zheng et al. loaded 6.6~11.0%
21 and 2.7~5.5% ($W(\text{carbohydrate}) / W(\text{conjugate}) * 100\%$) of GM3 onto KLH and BSA,

1 respectively. The sera obtained from mice immunized with the KLH-conjugated vaccines
 2 showed a significant titer of both IgM and IgG antibodies against GM3-HSA in ELISA assays.
 3 This indicates the occurrence of a GM3-specific immune response without other signals from
 4 the carrier KLH [97]. Guo's team invented a convergent synthesis method for the azido
 5 derivative of GM3, along with its convenient coupling to proteins using an acyl linker (Figure
 6 5C). This coupling strategy was efficient, with 10.8~13.5% and 11.9~17.5% of carbohydrate
 7 loading onto KLH and HSA, respectively. It was later confirmed that those KLH conjugates of
 8 GM3 and three derivatives, coupled using this method, induced a good GM3-specific antibodies
 9 in immunized mice after 35 days [98, 99].

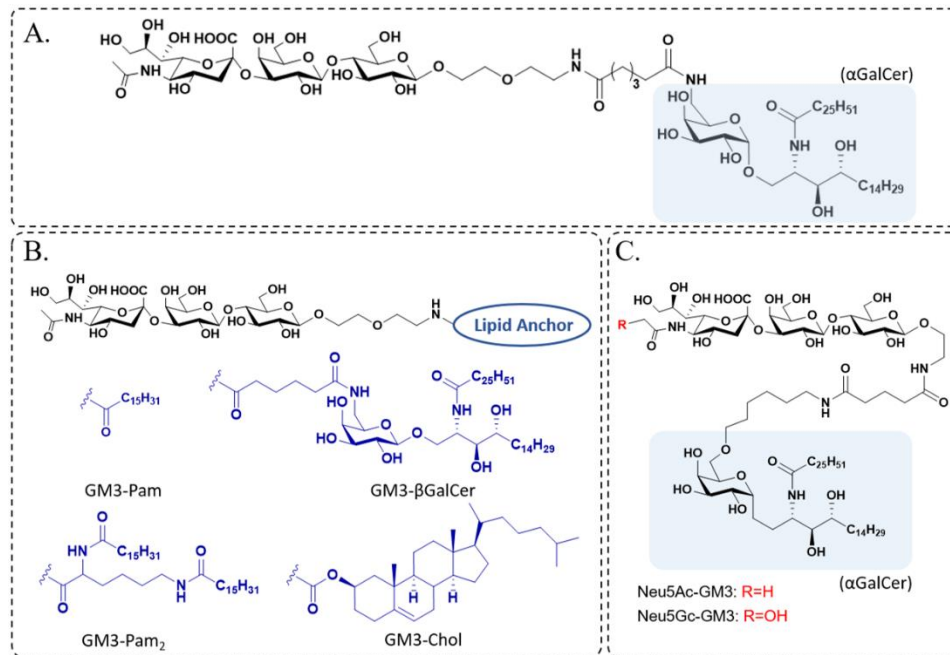


10 **Figure 5.** Representative linkers and general strategies for glycoconjugation to protein carriers. A. an
 11 example of a rigid cyclic 4-(maleimidomethyl) cyclohexane-1-carboxylate linker illustrating that
 12 complex linker structures will compete for immune responses, induce linker-specific antibodies and
 13 suppress TACA-specific antibody production; B. A general strategy for glycoconjugation with alkenyl
 14 glycosides. Reagents and conditions: (a) O₃ ozonolysis; (b) reductive amination reaction using
 15 NaBH₃CN. Details seen in Ref. 97; C. An azido group on reducing end of GM3 for neoglycoprotein
 16 coupling strategy. Reagents and conditions: (a) selective reduction of the azido group; (b)
 17 (CH₂=CH(CH₂)_nCO)₂O; (c) O₃ ozonolysis; (d) reductive amination using NaBH₃CN. Details seen in
 18 Ref. 98.

1 Several highly immunogenic recombinant proteins have also been employed in the
2 development of GM3 neoglycoconjugates and validated for anti-tumor vaccine activity [100].
3 For instance, Stepanenko's team utilized flagellins from Gram-positive bacteria (52.5 kDa,
4 containing 30 lysine residues) to couple with GM3, with an average of 11 GM3 molecules
5 coupled to each of this neoglycoconjugate. The C57Bl/6J mice were administrated with 2 doses
6 in an interval of two weeks. ELISA testing revealed a successful evocation of anti-GM3 IgG
7 and IgM antibody production, which is similar to those induced by the GM3-KLH vaccine.
8 Furthermore, dendritic cells pulsed with GM3-flagellins or GM3-KLH were used for mouse
9 immunization, and the flow cytometry analysis showed that this neoglycoconjugate induced
10 stronger MHC-II expression and CD80/CD86 differentiation [101, 102].

11 5.1.2 Agonist for iNKT cells

12 As an iNKT cell agonist, α GalCer has been utilized in the development of a self-adjuvant
13 vaccine based on GM3. In a study conducted by Yin et al., GM3 was covalently coupled to
14 α GalCer (Figure 6A). Besides, loaded with 4 different lipid linkers, GM3-lipids non-covalently
15 self-assembled with α -GalCer to form vaccine candidates (Figure 6B). Those chemical
16 synthesized vaccines were assembled with 1,2-distearoyl-sn-glycero-3-phosphocholine and
17 cholesterol to form liposomes. With immunization to BALB/c mice, these liposome vaccines
18 were evaluated according to anti-GM3 antibody production as well as the complement
19 dependent cytotoxicity assay (CDC) against the B16F10 cell line. The results demonstrated that,
20 the GM3- α GalCer covalent vaccine candidate can elicit a high level of anti-GM3 IgG
21 production, demonstrating potent binding affinity to cancer cells and effective activation of the
22 complement system. This underlined the importance of covalent linkers in enhancing cancer
23 vaccine efficacy. Although GM3 non-covalently conjugated to α GalCer through four lipid
24 linkers had higher GM3 loading ratios to the liposome vaccines in this study, the results
25 confirmed that these non-covalently coupled formulations were not as effective as GM3-
26 α GalCer [103].



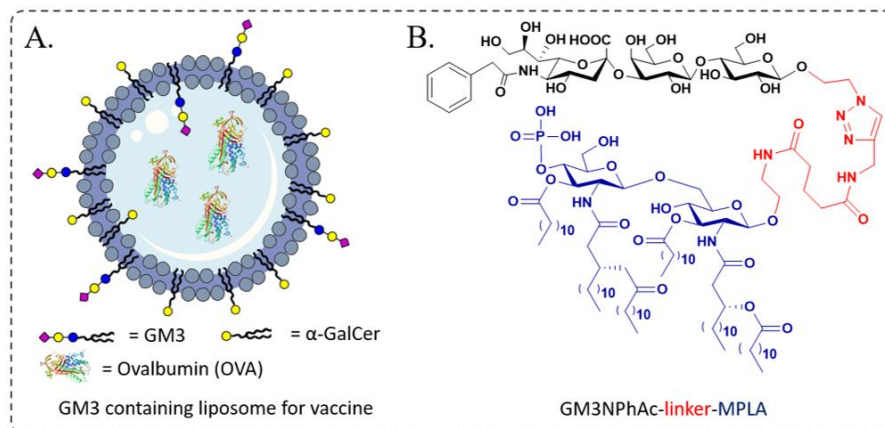
1 **Figure 6.** GM3- α GalCer vaccines, of which the α GalCer (in blue frame) performs the role of iNKT
 2 agonist and also an anchor for composition of liposome vaccine. **A.** A covalent GM3- α GalCer (self-
 3 adjuvant) vaccine candidate; **B.** Four non-covalent GM3-lipid/ α GalCer vaccine candidates. The four
 4 compounds were severally mixed with GalCer and self-assembled into liposomal structures by
 5 amphiphilicity. Details of A and B seen in Ref. 103. **C.** Two GM3- α GalCer vaccine candidates. Details
 6 seen in Ref. 104.

7 Romanò et al. reported a straightforward and efficient chemical synthesis method, covalently
 8 coupling Neu5Ac-GM3 and Neu5Gc-GM3 with α GalCer through a linker to form liposome
 9 vaccines (Figure 6C). These uniform liposomes with a controlled size of approximately 200nm
 10 were subjected to *in vitro* and *in vivo* immunological tests on iNKT cells and C57BL/6 mice,
 11 respectively. APCs pulsed with these vaccines were co-cultured with iNKT cells for 48 hours,
 12 a significant increase in the cytokines IL-4 and IFN- γ was observed in the *in vitro* culture
 13 medium. Similarly, murine immunization experiments also demonstrated that both vaccines
 14 could successfully activate the cytokines IL-4, IFN- γ , and IL-12p70, thereby recruiting Th1 and
 15 Th2 T-cell responses. Furthermore, they successfully activated B-cell responses, resulting in
 16 the production of anti-GM3 antibodies. The two sera induced by Neu5Gc-GM3 and Neu5Ac-
 17 GM3 vaccines, respectively, exhibit cross-reactivity against both antigens [104]. This
 18 phenomenon is not surprising in TACA vaccine research [105, 106].

19 In addition to coupling the trisaccharide moiety of GM3 to an immunogenic carrier, the
 20 complete GM3 ganglioside can also activate immune responses in the form of liposomes.

1 Haan's team reported a GM3 vaccine study based on the iNKT cell pathway. In this study, GM3
 2 ganglioside, α GalCer and OVA were mixed to form liposomes and used for mouse
 3 immunization (Figure 7A). Using CD169⁺ macrophage-deficient mice as a control, it was
 4 confirmed that this liposomal vaccine could activate downstream iNKT cells through antigen
 5 presenting by CD169⁺ macrophages. Furthermore, the liposome immunization group exhibited
 6 stronger cytokine production, CD8⁺ T-cell activation and B-cell responses (antibody production)
 7 compared to the GM3 ganglioside plus adjuvant group. This study suggests that GM3/ α GalCer
 8 liposome can serve as a platform promoting interactions between different immune cell
 9 populations, leading to robust adaptive immunity and emerging as a promising anticancer
 10 vaccination strategy [107]. Following this, the team reported a more mature GM3 antigen-
 11 containing liposome vaccine platform: the incorporation of TLR4 or TLR7/8 ligands promotes
 12 dendritic cell maturation and phagocytosis, leading to a stronger T-cell response [108].

13 In recent years, researches indicated that Neu5Gc-GM3 can bind to the CD1d receptor in
 14 both humans and mice [109, 110]. Furthermore, a study that published in 2016 demonstrated
 15 that Neu5Gc-GM3 can be presented by CD1d of B cells and activate iNKT cell responses. This
 16 discovery of Neu5Gc-GM3 serving as an iNKT cell agonist unveils an important
 17 immunological mechanism, expanding the potential functions of GM3 in immunotherapy and
 18 vaccine development [111].



19 **Figure 7.** A. A model of liposome formed by GM3 and α GalCer for vaccine delivery. Details
 20 seen in Ref. 107; B. A GM3 derivative, GM3NPhAc, conjugated with a toll-like receptor (TLR)
 21 ligand, mono-phosphoryl lipid A (MPLA) to form a vaccine candidate. Details seen in Ref. 122.

22 5.1.3 Pattern recognition receptors (PRRs) as carriers or co-delivery with liposomes

23 Toll-like receptors (TLRs) are a class of transmembrane PRR proteins typically found on

1 sentinel cells like macrophages and DCs. These receptors identify structurally conserved
2 molecules originating from microbes. Once these TLR ligand molecules encounter physical
3 barriers, they are firstly recognized by TLRs, initiating immune cell responses. Consequently,
4 TLR ligands can be employed as carriers for the development of TACA based anticancer
5 vaccines [112].

6 In early stages, researchers hydrolyzed the bacterial *N. meningitidis*, obtaining its cell wall
7 components known as *Neisserial* proteoliposomes. This is an immunogenic proteosome
8 capable of activating PPRs and self-assembling into proteoliposomes [113]. In order to boost
9 the immunogenicity, GM3 was combined with *Neisserial* proteoliposomes to produce very
10 small size proteoliposomes (VSSP). When utilized as a vaccine emulsified with an oil-based
11 adjuvant, the GM3/VSSP triggered robust anti-GM3 antibody titers (both IgM and IgG) in mice,
12 chickens, and monkeys [13]. Furthermore, GM3/VSSP administration demonstrated efficacy in
13 protecting against melanoma, as evidenced with highly anti-GM3 specific antibody production
14 and anti-B16 complement-mediated cytotoxicity in C57Bl/6 mice [114, 115]. Another research
15 confirmed that the GM3/VSSP vaccine induces dendritic cell maturation and presentation of
16 GM3 through TLR2-mediated signal transduction, activating the release of IL-6 and IFN- γ
17 cytokines [116]. In a Phase I clinical trial involving 26 participants with metastatic melanoma,
18 where GM3/VSSP was utilized as an anti-melanoma vaccine, 44% of the subjects exhibited the
19 production of anti-GM3 IgM in their serum. Moreover, these sera were observed to elicit
20 responses against melanoma cell lines and biopsy tissues. A robust secretion of IFN- γ cytokine
21 was detected in the sera of all participants, with one sample identified as GM3-specific [117].

22 With the development of synthetic chemistry, the core structures derived from bacterial LPS
23 have been gradually elucidated and successfully synthesized [118, 119]. The mono-phosphoryl
24 lipid A (MPLA) is 1-*O*-dephosphorylated lipid A, which is a most representative bacterial LPS
25 structure. MPLA exhibits potent immunostimulatory properties and serve as effective vaccine
26 adjuvants. The immunostimulatory activity of MPLAs is ascribed to their capacity to initiate
27 cytokine cascades by interacting with toll-like receptor 4 (TLR4). This interaction can trigger
28 antigen presentation, activate T-helper (Th) cells, and consequently induce T cell-mediated
29 immunity [120]. Guo's team identified a highly immunogenic GM3 derivative trisaccharide
30 GM3NPhAc in their former study [99, 121]. Based on this, the *N. meningitidis* MPLA was used

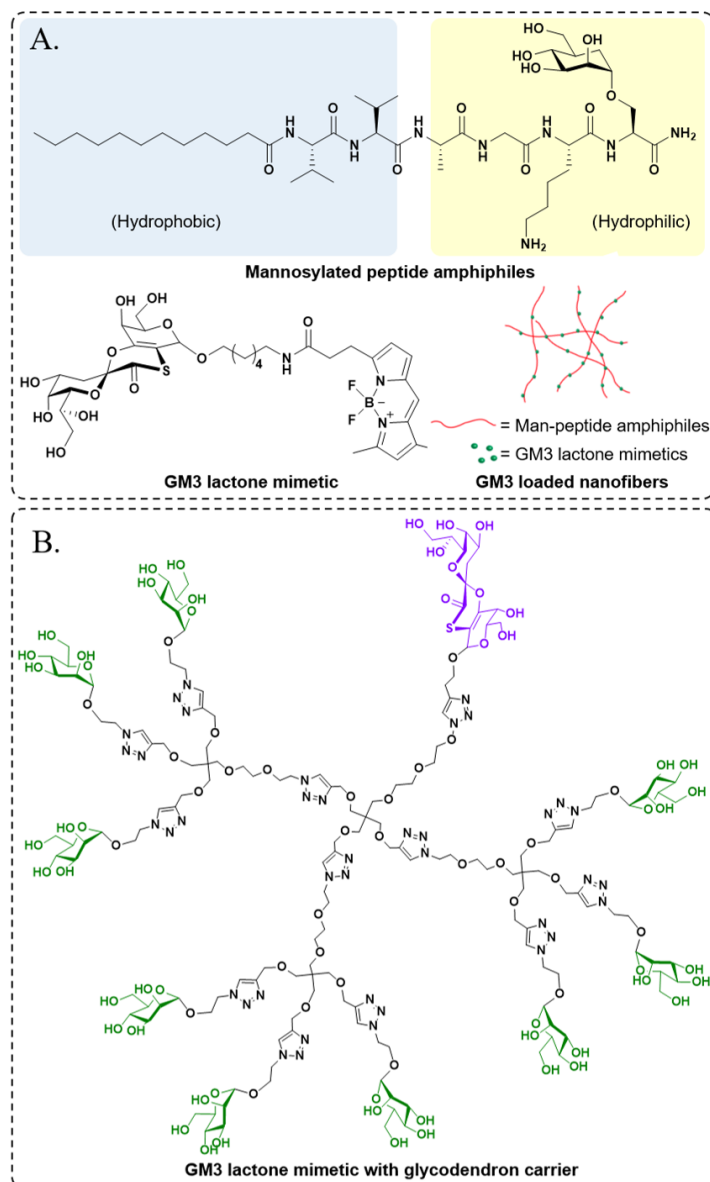
1 as vaccine carrier to create a glycoconjugate and administered to mice (Figure 7B). The results
2 revealed that this vaccine elicited an effective immune response to GM3NPhAc without the use
3 of an external adjuvant, particularly leading to the generation of carbohydrate-specific IgG3
4 subtype antibodies. Interestingly, for one test group in this study was utilized Titermax Gold as
5 adjuvant, but it was observed that the adjuvant did not enhance the antibody production; instead,
6 it appeared to inhibit the GM3NPhAc-specific immune response. This provides insights for
7 selecting carriers and immunization strategies in the development of GM3 anti-tumor vaccines
8 [122].

9 In addition to serving as an antigen, GM3 can also be in co-delivered with PRRs by liposomes
10 and facilitate macrophage to capture the liposome vaccines. CD169⁺ splenic macrophages
11 efficiently capture particulate antigens from the blood and transfer them to dendritic cells for
12 downstream immune responses. In a study published in 2020 by Haan's group, it was reported
13 that the inclusion of GM3 in liposomes enhances the engulfment by CD169⁺ splenic
14 macrophages, thereby presenting antigens to activate CD8⁺ and CD4⁺ T cell responses [123].
15 The team reported new results two years later, with a study that they incorporated MPLA (TLR4
16 ligand), 3M-052 (TLR7/8 ligand) and an inflammasome stimulus either alone or in combination
17 into GM3 liposomes. Both *in vitro* and *in vivo* results confirmed that MPLA-containing GM3
18 liposomes are effective vectors to induce DC maturation and CD4⁺/CD8⁺ T cell priming.
19 Although these studies did not focus on whether the immunized animals produced a specific
20 anti-GM3 response, the activation of macrophages by GM3 liposomes itself provides a
21 promising strategy for the development of anti-tumor vaccines [108].

22 5.1.4 Other carriers

23 Other emerging highly immunogenic biomaterials have also been developed as carriers for
24 coupling TACAs to prepare vaccines. For instance, Virus-like particles (VLPs) are complex
25 protein structures that mimic the envelopes or capsids of authentic native viruses but lack the
26 viral genome, falling within a similar particle size range (0.02–0.06µm) [124]. Taking
27 advantage of this more natural immunogenic structure, VLPs have been developed for TACA
28 delivery. Huang's group utilized a cowpea mosaic virus (CPMV) capsid as the immunogenic
29 carrier for conjugation with Tn antigen. The resulting Tn-CPMV was administered to mice,

1 generating high total antibody titers and high IgG titers specific to Tn antigen [125]. In another
 2 study, a bacteriophage Q β conjugated with SLewis^a antigen was used to immunize mice.
 3 Compared to the control group immunized with SLewis^a-KLH, the sera from SLewis^a-Q β
 4 immunized mice exhibited higher anti-SLewis^a antibody titers, which remained detectable at
 5 high levels even after 379 days [126].



6 **Figure 8. A.** Nanofibers loaded with GM3 lactone mimetic as a vaccine candidate. Details
 7 seen in Ref. 127; **B.** Structure of a glycodendron consisting of nine mannoses (in green) for
 8 DC targeting and one GM3 lactone mimetic (in purple) as antigen. Details seen in Ref. 128.

9 In recent years, with highly regulated dimensions, controllable volumes and abundant surface
 10 modification sites, nanomaterials have also been developed as chemical carriers for TACA
 11 based vaccines, e.g., gold nanoparticles, nanofibers and other chemical scaffolds. Nativi's group

1 synthesized a thioether-bridged mimetic of GM3 lactone for antigen coupling with various
2 nanomaterial carriers [51, 127-129]. They synthesized a mannose-containing peptide
3 amphiphiles, which can spontaneously assemble into nanofibers in aqueous solution with
4 diameters on the order of 9~11 nm and lengths reaching several micrometers. These nanofibers
5 were then mixed with a hydrophobic fluorescent GM3 lactone mimetic, allowing the
6 hydrophobic portion to be inserted into the interior of the nanofiber while exposing the
7 hydrophilic antigenic portion outside (Figure 8A). The abundant mannose on this nanofiber
8 carrier serves as a ligand for DC-SIGN on DCs. By *in vitro* experiments, it was confirmed that
9 these nanofibers could activate DCs to phagocytize the antigens of GM3 lactone mimetic.
10 Furthermore, the over-expression of CD86, CD83 and MHC-II on DCs indicated that these
11 nanofibers could also promoted the maturation of DCs [127]. Similarly, they synthesized a
12 bifunctional multivalent glycodendron consisting of nine mannose residues for DC targeting
13 and one residue of the antigen, GM3 lactone mimetic (Figure 8B). Immunological assays
14 revealed that this glycodendron is able to trigger the activation of human immature DCs and
15 promote the proliferation of T lymphocytes in a mixed lymphocyte reaction [128].

16 **5.2 Chemical modifications on GM3**

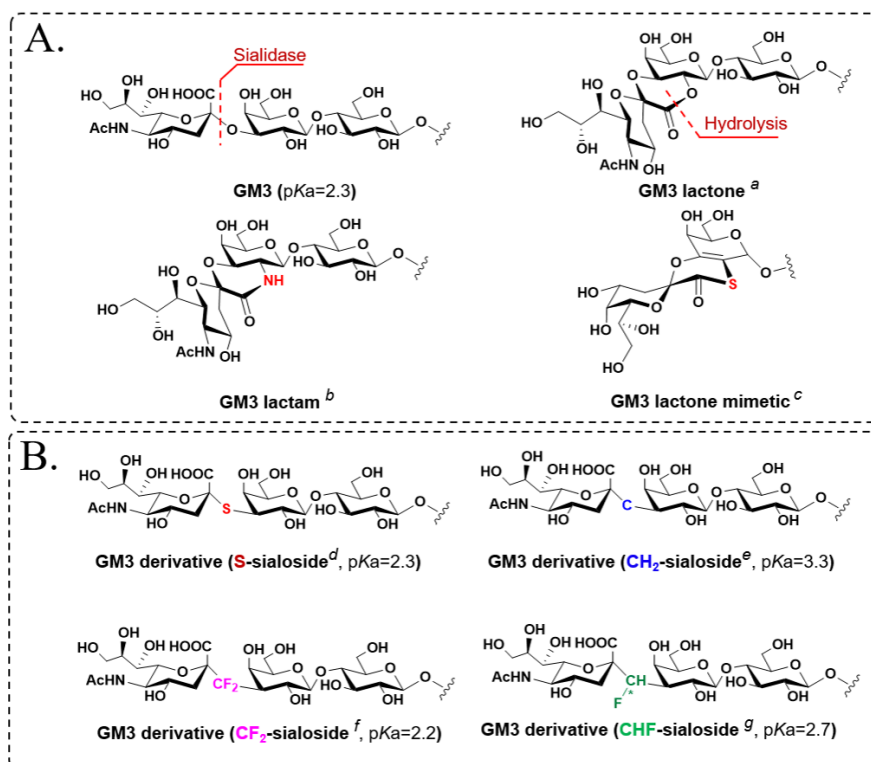
17 Protein-carbohydrate interactions present peculiarities compared to general protein-rigid
18 ligand and protein-peptide interactions. Firstly, many carbohydrates exhibit extreme flexibility
19 and contain numerous hydroxyl groups, resulting in the formation of extensive hydrogen-
20 bonding networks that significantly influence the overall binding free energy. Secondly, a
21 notable characteristic of protein-carbohydrate interactions is the formation of essential CH- π
22 contacts between the aromatic side chains of the protein and the C-H bonds of the carbohydrates,
23 particularly on their hydrophobic surfaces [130, 131]. Therefore, the specificity in
24 carbohydrate-antibody interactions is more tolerant. Taking GM3 as an example, numerous
25 studies in recent years have demonstrated that chemically modified GM3 derivatives exhibit
26 enhanced immunogenicity during the immune response, and the resulting antibodies show
27 varying degrees of cross-reactivity to natural GM3, which means the antibodies induced by
28 derivatives could fight against the GM3 containing cancer cells [132]. This provides an
29 important strategy for the development of GM3 derivatives as anti-tumor vaccines.

1 5.2.1 GM3 lactone and mimetics

2 As early as 1987, Hakomori's team found that a monoclonal antibody M2590 reacts not only
3 with GM3 but also with GM3 lactone (Figure 9A-a), and the antibody exhibits a significantly
4 higher binding affinity to GM3 lactone compared to GM3. Based on this finding, they
5 immunized BALB/c mice separately with GM3 and GM3 lactone. The results showed that GM3
6 lactone is more immunogenic, triggering a stronger production of antibodies which were able
7 to bind to both GM3 and GM3 lactone demonstrating cross-reactivity to both antigens [43].
8 Subsequently, they immunized C57BL/6 mice with GM3 lactone coated on *Salmonella*
9 *minnesotae*, establishing hybridoma DH2 which secretes IgG3 subtype antibodies. Those
10 antibodies were proven to preferentially react with GM3 lactone over GM3, but inhibited
11 melanoma cell growth *in vivo* and induced antibody-dependent cytotoxicity *in vitro* [133].

12 While GM3 lactone exhibits higher immunogenicity compared to natural GM3, the stability
13 of the lactone's ring structure remains a concern. To overcome the hydrolytic instability of the
14 lactones, Magnusson and colleagues developed a GM3 lactam (Figure 9A-b) which should be
15 hydrolytically stable *in vivo* while still being conformationally similar to the corresponding
16 lactone structure. Immunization of BALB/c mice with a GM3 lactam-BSA conjugate, followed
17 by the generation of hybridomas, resulted in a significant number of clones specific to GM3
18 lactam. Intriguingly, the majority of these clones exhibited recognition of GM3 lactone but not
19 GM3 [134].

20 For the same purpose of enhancing hydrolytic stability, Nativi's team synthesized a thioether-
21 bridged mimetic of GM3 lactone (Figure 9A-c) and conjugated it with various carriers for
22 vaccine biological activity testing [135]. In their work published in 2010, this mimetic was
23 conjugated to KLH and used to immunize BALB/c mice. A hybridoma producing the strongest
24 anti-GM3 antibody was selected, and further analysis showed its isotypes were identified as
25 IgM and IgG3. Immunocytochemistry results demonstrated that polyclonal antibodies from the
26 immunized mouse serum bound to HCT116 cells (human colon tumor cells overexpressing the
27 GM3 antigen) [51]. Subsequently, in 2014 and 2017, they respectively reported the conjugation
28 of this mimetic of GM3 lactone to non-protein carriers glycodendron and nanofibers as vaccine
29 candidates, successfully activating DC immune response.



1 **Figure 9. A.** GM3 lactone and mimetics (red dashed lines indicate chemical bond susceptible to sialidase
 2 or hydrolysis). Details seen in ^aRef. 43, 133, ^bRef. 134, and ^cRef. 135; **B.** Sialidase-resistant analogues of
 3 GM3. Details seen in ^dRef.137 and ^eFigRef. 140.

4 5.2.2 Modifications on the glycosidic bond

5 Sialidases are enzymes that can cleave sialic acid glycosidic bonds. For example, the
 6 upregulated sialidase NEU3 in many malignant cancers can cleave *O*- α -2,3 glycosidic bonds
 7 and remove sialic acid from GM3. The exogenous GM3 introduced as anticancer medicines or
 8 vaccines needs to avoid degradation by these glycosidases and maintain its intact structure to
 9 exert biological activity. Therefore, many studies have developed representative sialidase-
 10 resistant active analogues targeting sialic acid glycosidic bonds.

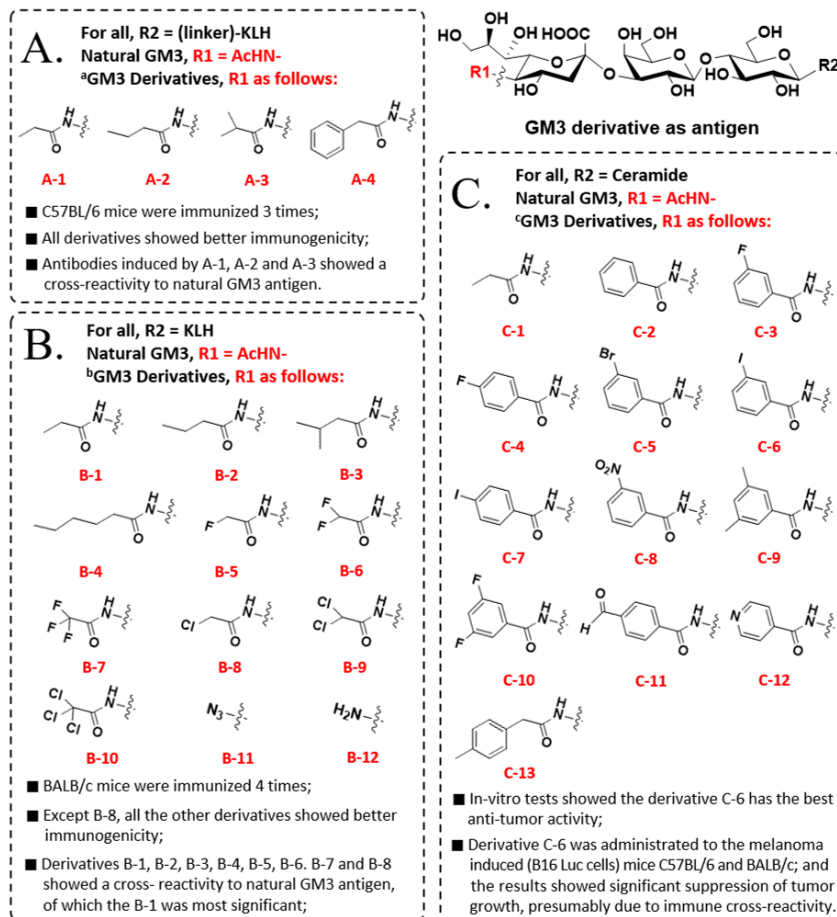
11 One direct method involves substituting the susceptible *O*-sialoside bond in α -2,3-
 12 sialylgalactose with an alternative linkage, while minimizing structural alterations to the cyclic
 13 sialic acid moiety. Considering this, *S*-Sialosides (Figure 9B-d) was taken into tests, which
 14 exhibit greater flexibility compared to *O*-sialosides due to their longer C-S bond, leading to
 15 decreased steric and stereo-electronic effects [136]. Bundle's group synthesized this *S*-sialoside
 16 substituted GM3 analogue and loaded it onto TTox for immunological assays. Five BALB/c
 17 mice were immunized with three doses of *S*-GM3-TTox glycoconjugates on days 1, 21 and 28,
 18 and serum antibodies were tested on day 38. The results showed a strong antigen-specific IgG

1 response when immune sera were reacted against *S*-GM3. Additionally, three out of five mice
2 showed a cross-reactivity against natural GM3, with good antibody titers ranging from 10^3 to
3 10^4 . Analysis of IgG subtypes indicated that this *S*-GM3 glycoconjugate vaccine successfully
4 activated the T cell-dependent immune pathway [137, 138]. The analysis of molecular
5 structures suggests that while the C-S bond is longer than the C-O bond, the C-S-C angle is
6 significantly smaller than the C-O-C angle. *S*-glycosidic bonds, being less constrained than
7 their *O*-glycoside counterparts, can more readily access the higher energy anti conformation in
8 both unbound and bound states. The replacement of oxygen with sulfur atom leads to longer
9 bonds and weaker stereo-electronic effects, allowing for substantially greater flexibility. This
10 flexibility poses a potential challenge to the use of *S*-glycoside immunogens [139]. According
11 to the *in vivo* experimental results, it appears that this flexibility introduces some randomness
12 but still holds significant inspiring implications.

13 Another category of glycosidic modification for sialidase-resistance of GM3 analogue that
14 has attracted attention is *C*-glycosides. Harai's group has recently incorporated four types of *C*-
15 sialoside linkages (CH_2 -, CF_2 -, (*R*)-CHF-, and (*S*)-CHF-sialosides, shown in Figure 9B-e, f and
16 g) into the synthesis of GM3 using a series of strategies [140]. They first assessed the impact
17 of each glycoside on the *pKa* value of sialic acid in these *C*-sialosidic analogues. The results
18 showed that the CH_2 -sialoside could increase the *pKa* of sialic acid (from 2.3 to 3.3,
19 theoretically). Furthermore, in the formation of the *O*- α 2,3 linkage, the lone pairs electrons on
20 the oxygen atom can achieve a more stable conformation based on the exo-anomeric effect. In
21 contrast, the CH_2 -sialoside lacks stereo-electronic effects, resulting in greater conformational
22 flexibility. Therefore, they considered introducing fluorine atoms to maintain the *pKa* of sialic
23 acid while obtaining relatively stable conformation of the GM3 analogues through the gauche
24 effect. *In vitro* experiments confirmed that these fluorinated *C*-glycosidic GM3 analogues
25 exhibited biological activity similar to natural GM3 by inhibiting the epidermal growth factor
26 receptor. Additionally, the (*S*)-CHF-linked GM3 analogue with a stable exo-gauche
27 conformation showed the strongest activity in promoting Had-1 cell growth, indicating the
28 importance of the specific conformation of GM3 for the biological functions [136]. These
29 findings suggest promising avenues for research into the immunological activity of such *C*-
30 glycosidic GM3 analogues in anticancer vaccine development.

1 5.2.3 Modifications on sialic acid of GM3

2 Taking advantage of the potential cross-reactivity in antibody-carbohydrate reactions,
 3 researchers have introduced chemical modifications to sialic acid in order to disrupt the
 4 immunotolerance and immunosuppression of GM3. Guo's team replaced the acetamido group
 5 of sialic acid and synthesize four GM3 derivatives A-1~A-4 as shown in Figure 10A. The
 6 immunological properties of the GM3 derivative-KLH glycoconjugates were assessed in
 7 C57BL/6 mice with 3 immunizations at day 1, day 27 and day 35. By analyzing the serum
 8 collected on day 35 using ELISA, the results showed that GM3-KLH only activated an IgM
 9 response, while the other four GM3 derivative-KLH conjugates not only activated an IgM
 10 response but also elicited a stronger IgG response. Overall, glycoconjugate A-4 exhibited the
 11 highest immunogenicity, followed by A-3, A-2, and A-1. However, only sera from mice
 12 immunized with A-1, A-2, and A-3 showed cross-reactivity with natural GM3. These findings
 13 suggest that larger structural modifications of sialic acid (e.g. A-4) can improve
 14 immunogenicity, but the induced antibodies may not react against natural GM3 due to the
 15 significant differences in the antibody binding pockets [99].



16 **Figure 10.** GM3 derivatives with N-modification on sialic acid. Details seen in ^aRef. 99, ^bRef. 97 and ^cRef. 142.

1 Taking lessons from their previous work, Ye et al. found that the carboxylic acid and sugar
2 type are more sensitive to maintain the cross-recognition of antibodies induced by TACA
3 derivative and should be kept intact [141]. They focused on maintaining the carboxyl group of
4 sialic acid unchanged and instead pursued *N*-acyl modification. Twelve structurally modified
5 GM3 derivatives and their KLH conjugates (B-1~B-12 shown in Figure 10B) were designed
6 and synthesized. BALB/c mice were immunized four times at 2-week intervals with GM3-KLH
7 or GM3 derivative-KLH glycoconjugates. After the third vaccination, pooled sera were
8 analyzed for IgG titers against each derivative antigen or natural GM3. Except B-8, all the other
9 modified GM3 derivatives exhibited stronger immunogenicity, eliciting higher titers of
10 antibodies against their corresponding GM3 derivative antigens compared to unmodified GM3.
11 Due to concerns about cross-reactivity, this study found that glycoconjugates B-1, B-2, B-3, B-
12 4, B-5, B-6, B-7 and B-8 elicited antibodies cross-reacting against GM3. Among them, B2 and
13 B7 elicited higher titers of IgG antibodies against GM3 compared to the unmodified GM3-KLH
14 [97].

15 In an article published in 2022, Jiang et al. synthesized 13 *N*-acyl modified GM3 derivatives
16 (C-1~C-13 shown in Figure 10C) and evaluated their anti-tumor activity by wound healing
17 assays and trans-well assays. Among these derivatives, compound C-6 exhibited significant
18 inhibition of cell migration and invasion, as well as marked anti-tumor effects in tumor-bearing
19 (epidermoid carcinoma A431 cells) C57BL/6 mouse model. Subsequent analysis of cancer
20 tissues and serum samples revealed that compound C-6 prompted tumor suppression through
21 inducing tumor necrosis and eliciting an inflammatory response, indicating that the tumor
22 inhibition induced by C-6 was closely associated with the carbohydrate cross-reactivity
23 immune response [142]. Although this hypothesis is not entirely consistent with the previous
24 findings conducted by Guo regarding glycoconjugate A-4 (GM3NPhAc-KLH), where the
25 introduction of a large phenyl structure to *N*-acyl moiety of GM3 did not elicit cross-reactivity
26 against natural GM3, the promising anti-tumor activity exhibited by compound C-6 still
27 warrants further investigation into immunological pathways.

28 5.2.4 Vaccines based on metabolic glycoengineering of GM3

29 While antibodies generated by GM3 derivatives with more complex modifications (e.g. *N*-

1 phenylacetyl modification on sialic acid) are unlikely to cross-react with natural GM3, the
 2 significantly increased immunogenicity still attracts researchers to develop anticancer
 3 immunotherapies using them. Glycoengineering based on sialic acid metabolism could provide
 4 a highly specific immunotherapy strategy to overcome this issue. In simple terms,
 5 glycoengineering is a process based on cellular metabolism where sialic acid is synthesized
 6 from mannose substrates and catalyzed by sialyltransferase to oligosaccharides, which are
 7 further transferred to the cell surface (Figure 11) [143, 144]. By utilizing this pathway, the
 8 introduction of chemically modified *N*-acetyl mannose (ManNAc) can introduce more
 9 corresponding modified sialyl TACAs, including GM3, onto the surfaces of cancer cells. This
 10 strategy was pioneered by Reutter and colleagues [145]. Further advancements in bioorthogonal
 11 chemistry, inspired by the groundbreaking work of Bertozzi and colleagues, and led to Bertozzi
 12 being awarded the Nobel Prize in Chemistry in 2022 [146]. Given that this glycoengineering
 13 enables the editing of cancer cell surfaces with modified GM3, the antibodies activated by the
 14 highly immunogenic GM3 derivatives mentioned earlier now have a role to play. These robust
 15 immune responses can selectively target the glycoengineered cancer cells.

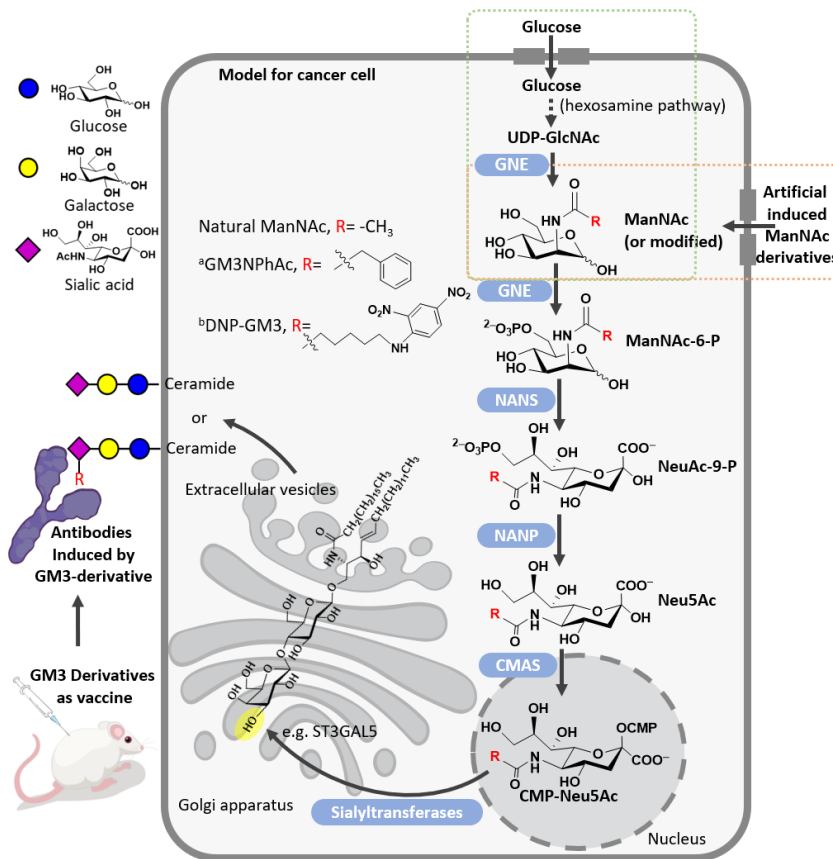


Figure 11. Vaccines based on metabolic glycoengineering of GM3 and an overview of

1 mammalian sialic acid metabolism shown in a cellular sketch. Starting from the top, normally the
2 glucose is taken by cancer cells and converted into ManNAc by following the hexosamine
3 pathway and GNE (pathway details seen in Ref. 144). If a ManNAc with *N*-modification is
4 artificially introduced into the cellular environment, it will participate in the subsequent pathway:
5 catalyzed by enzymes such as GNE, NANS, NANP, and CMAS (pathway details seen in Ref.
6 143), ultimately transforming into *N*-modified sialic acid. These sialic acids are then added onto
7 GM3 by sialyltransferases such as ST3GAL5, and transported to the cell membrane surface via
8 vesicles. Antibodies and immune responses elicited by immunization with the corresponding
9 GM3 derivative will specifically recognize these glycoengineered cancer cells. Details seen in
10 ^aRef. 147 and ^bRef 149.

11 The GM3NPhAc (shown as Figure 11. a) glycoconjugate elicited a significantly strong anti-
12 GM3NPhAc-specific antisera production but the antibodies hardly showed cross-reactivity to
13 natural GM3 [99]. In a subsequent study, tumor cells (K562, SKMEL-28, and B16-F0) were
14 incubated with *N*-phenylacetyl-mannosamines (ManNPhAc), resulting in successful
15 glycoengineering as identified by flow cytometry. These glycoengineered cancer cells exhibited
16 sensitivity to anti-GM3NPhAc-specific antisera in CDC assays, indicating the potential of this
17 strategy for developing glycoengineering-based anti-tumor vaccines [147]. In 2015, their team
18 applied this strategy to animal models and obtained promising results. C57BL/6 mice
19 immunized with GM3NPhAc-KLH-loaded DCs successfully activated T cell-dependent anti-
20 GM3NPhAc-specific immune responses. One week after the third immunization, mice were
21 injected with 5×10^5 of B16F10 cells followed by daily injections of ManNPhAc (50 mg/kg/day)
22 for 7 days. The results demonstrated that this strategy significantly inhibited tumor growth and
23 prolonged the survival of tumor-bearing mice, while also reducing B16F10 lung metastases
24 [148].

25 In a recent study, GM3 was introduced with a more complex *N*-modification for the aim of
26 eliciting a stronger immunogenic response through pre-immunization to the anti-modification
27 and employing glycoengineering strategies for anti-tumor vaccine therapy. A 2,4-dinitrophenyl
28 (DNP)-modified GM3 intermediate (DNP shown as Figure 11. b) was chemoenzymatically
29 synthesized and conjugated to KLH, conducted by Wu's group. BALB/c mice pre-immunized
30 with DNP-OVA were subsequently immunized with the DNP-GM3-KLH glycoconjugates.
31 This approach resulted in a robust and rapid immune response, attributed to the recruitment of
32 anti-DNP antibodies facilitating targeted delivery of the vaccine construct to APCs. The serum

1 obtained through this immunization strategy consisted of IgG subtype antibodies without IgM,
2 and exhibited significant CDC against Man-NHDNP treated B16F10 melanoma cells *in vitro*
3 [149].

4 **6. Conclusion**

5 Great progress has been made in the past decades for carbohydrate-based anticancer vaccine
6 development. As a member of TACAs, the structure and distribution of GM3 molecules make
7 them a prominent target for the development of anti-tumor immunotherapy. Various strategies
8 have been attempted to exploit its potential in anti-tumor immunotherapy, including activating
9 the immune response to GM3 hapten through immunogenic carriers, enhancing
10 immunogenicity by modifying the GM3 glycoside portion then for cross-reactivity with natural
11 GM3, and introducing labeled GM3 onto tumor cells through glycoengineering-based bio-
12 orthogonal reactions. These strategies collectively demonstrate the effectiveness of GM3 in the
13 development of anti-tumor immunotherapy. Based on these foundations, determining GM3
14 derivatives with higher immunogenicity and cross-reactivity to natural antigens, identifying the
15 most cost-effective synthesis methods, selecting the most efficient delivery carriers, and other
16 related tasks are all essential steps before advancing GM3 into clinical applications.

17 **CRedit authorship contribution statement**

18 Dr. Yongmin Zhang, Dr. Marco Terreni and Dr. Matthieu Sollogoub contributed to the
19 conceptualization, revising, and funding acquisition; Jiaxu Zhang contributed to the original
20 draft, preparation for the figures and tables, and editing; Fang Liu contributed to the data
21 collection and revising. All the authors participated in the discussions of the results.

22 **Conflict of Interest Statement**

23 The authors declare that they have no known competing financial interests or personal
24 relationships that could have appeared to influence the work reported in this paper.

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