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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)

4 **Authors and Institutes:**

5 Jiayu Zhang^{1,3}, Marco Terreni², Fang Liu¹, Matthieu Sollogoub¹, and Yongmin Zhang^{1,3*}

6 ¹Sorbonne Université, CNRS, Institut Parisien de Chimie Moléculaire, UMR 8232, 4 Place
7 Jussieu, 75005 Paris, France

8 ²Drug Sciences Department, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy

9 ³College of Life Sciences, Northwest University, Xi'an 710069, China

10 ***Correspondence to:**

11 Sorbonne Université, CNRS, Institut Parisien de Chimie Moléculaire, UMR 8232, 4 Place
12 Jussieu, 75005 Paris, France; Tel: 33-144276153; Fax: 33-144275504; E-mail:
13 yongmin.zhang@upmc.fr

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
18 acid 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**,
28 *N*-acetyl-glucosamine; **GalNAc**, *N*-acetyl-galactosamine; **Fuc**, fucose; **Neu5Ac**,
29 *N*-acetylneuraminic acid; **Neu5Gc**, *N*-glycolylneuraminic acid

1. Introduction

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

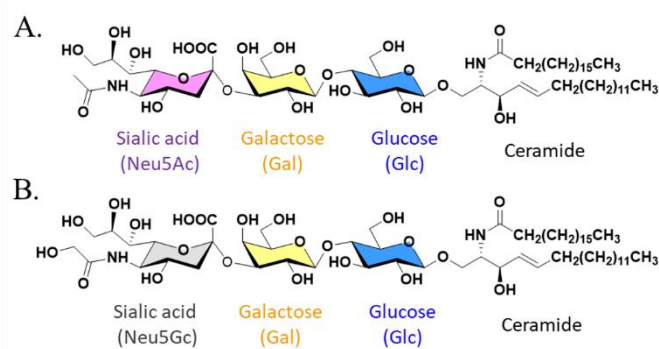


Figure 1. Representative structures of ganglioside GM3. **A.** Neu5Ac-GM3; **B.** Neu5Gc-GM3

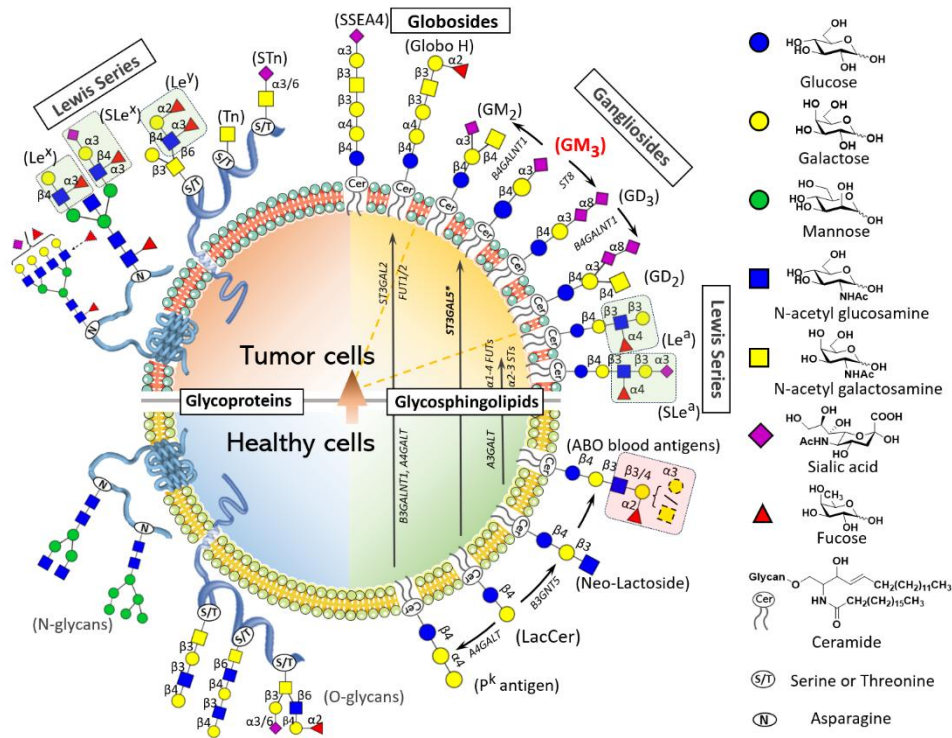
Ganglioside GM3 is over-expressed on some tumor cells, which makes it defined as a tumor-associated carbohydrate antigen (TACA). Studies have already identified that GM3 is significantly overexpressed in melanoma and not found in healthy skin or skin neighboring primary melanoma [6, 7], and the content of GM3 on cancer cells is positively correlated with the metastasis ability [8, 9]. It is worth mentioning that an important GM3 variant, Neu5Gc-GM3 (Figure 1B), was detected in melanoma, colon and breast cancer cells and was not present 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
3 hotspot. However, a major drawback of GM3 anticancer vaccines is the poor immunogenicity
4 (ability to induce immune response) and antigenicity (ability to bind immunoglobulins), due
5 to the 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
9 responses to the carbohydrate vaccines, this review summarizes the studies on the
10 development of GM3 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
15 *O*-glycosylated glycoproteins [17]. As shown in Figure 2, the *N*-glycans sharing a
16 penta-saccharide core structure ((Man)₃- (GlcNAc)₂-), while the most common
17 *O*-glycosylation is starting from *N*-acetyl-galactosamine (GalNAc). GSLs consist of a
18 hydrophobic ceramide (Cer) moiety anchoring the outer layer of cell membranes and a glycan
19 moiety extending into the extracellular matrix due to the hydrophilicity [18]. Generally, the
20 glycoside moiety of GSLs starts from glucose, and various monosaccharides extend and
21 branch the glycan structure under the action of glycosyltransferases to form diverse complex
22 oligosaccharides (e.g. p^k series, Neo-Lactosides, Globosides and Gangliosides) [19]. The most
23 representative human functional GSLs are the ABO blood group antigen system. These
24 carbohydrate antigens, present on red blood cells, differ by a monosaccharide residue at the
25 terminal end of the glycan, yet they impose significant limitations on blood transfusion
26 compatibility [20]. There is new evidence that mammals use RNA as a third scaffold for
27 glycosylation. Those so-called "glycoRNAs" which consisted of small noncoding RNAs and
28 sialylated glycans were present in multiple cell types and can interact with anti-dsRNA
29 antibodies and members of Siglec receptors [21]. This discovery will greatly promote

1 research in the fields of glycoscience and help scholars more clearly understand the important



2 role of glycans in life activities.

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

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

1 related glycoproteins [26]. One of the most thoroughly studied breast cancer associated
2 glycoprotein is mucin, which is a type I transmembrane glycoprotein with a unique
3 extracellular domain consisting of lots of tandem repeat of 20 amino acids
4 (HGVTSAPDTRPAPGSTAPPA) providing abundant Ser and Thr as potential sites for Tn or
5 STn *O*-glycosylation [27].

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

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

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

1 Ganglioside GM3 is widely present in virtually all types of animal cells and exhibits
2 over-expression in various human cancers, including melanomas, lung, and brain cancers [34,
3 35]. GM3 expression in melanomas has been extensively investigated. Reports indicate a
4 significantly higher percentage of GM3 existence in both primary and metastatic melanomas
5 compared to GD2 [36]. Additionally, there is a strong correlation between GM3 and GD3
6 expressions in both primary and metastatic melanomas. Notably, GM3 is absent in normal
7 skin and the skin adjacent to primary melanomas, in contrast to GD3. The onset of GM3
8 expression occurs at an early stage of melanocyte differentiation, facilitating entry into the
9 dermis of the skin [7, 37]. The detection of GM3 on the surface of tumor cells is a critical
10 determinant of the metastatic phenotype [38]. Cell line B16 is derived from the classic
11 melanoma mouse model C57BL/6, which is well established and widely used as tumor model
12 to study melanoma metastases, melanoma-specific immune cells reactions and
13 pharmaceutical activities [39]. Studies have indicated that GM3 density can influence
14 antigenicity. The anti-GM3 mAb M2590 interacts with GM3 over-expressed melanoma B16
15 cells while exhibiting no reactivity with corresponding normal tissue [40]. Therefore,
16 researchers generally believe that the densely packed cluster formed by GM3 on the
17 melanoma cell membranes is the key to the action of drugs or antibodies [41-43].

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

1 metabolically active tumor cells exhibit such an abnormal expression of Neu5Gc-GM3.

2 Another GM3 associated molecule, GM3 lactone, was also found in human and mouse
3 melanoma cells [43]. The carboxyl group on sialic acid undergoes condensation with the
4 2-hydroxyl group of the adjacent galactose, forming a lactone. This formation is likely
5 facilitated by the lower pH of TME within tumor cells and potentially influenced by a distinct
6 conformation of GM3 ganglioside induced on the tumor cell surface due to its local high
7 density [51]. The specificity of GM3 expression on tumor cells and the variant potential of the
8 molecule itself make them promising for the development of anti-tumor immunotherapy [52].

9 **4. Principles of glycoimmunology**

10 The development of vaccines against carbohydrates was first pursued to immunize against
11 bacterial pathogens. The surface of bacteria contains abundant lipopolysaccharide (LPS)
12 structures, of which the polysaccharides are generally rich in galactose and sialic acid [53, 54].
13 Mammals have an innate immune system with conserved pattern recognition receptors (PRRs)
14 that can recognize the certain glycopatterns from LPSs. For example, there are three major
15 classes of carbohydrate-recognized lectins as pathogen-associated molecular patterns
16 (PAMPs), galectins, Siglecs and C-type lectin receptors [55]. This is an early defense
17 mechanism against bacterial infections. In addition to this early protection, immunizing the
18 body with a certain dose of LPS antigen can activate a more specific humoral immune
19 response, produce corresponding immunoglobulin antibodies, and trigger a downstream
20 immune cascade. It is first time to report in 1917 that there was a specific soluble substance
21 which could induce antibodies for *Pneumococci*, and it was subsequently identified as
22 polysaccharide [56, 57]. Nowadays, vaccines based on bacterial LPSs have been well
23 developed and routinely used in an immunization schedule to prevent people from
24 *Haemophilus influenzae* type B, *Streptococcus pneumoniae* and *Neisseria meningitidis* [58,
25 59].

26 The application of bacterial LPS vaccines has confirmed that the effectiveness of glycans
27 or glycopatterns are immunogenic substances. Abnormal glycosylation and their
28 corresponding product TACAs become hallmarks of cancer and can serve as a source of novel
29 clinical biomarker developments, providing a set of specific targets for therapeutic

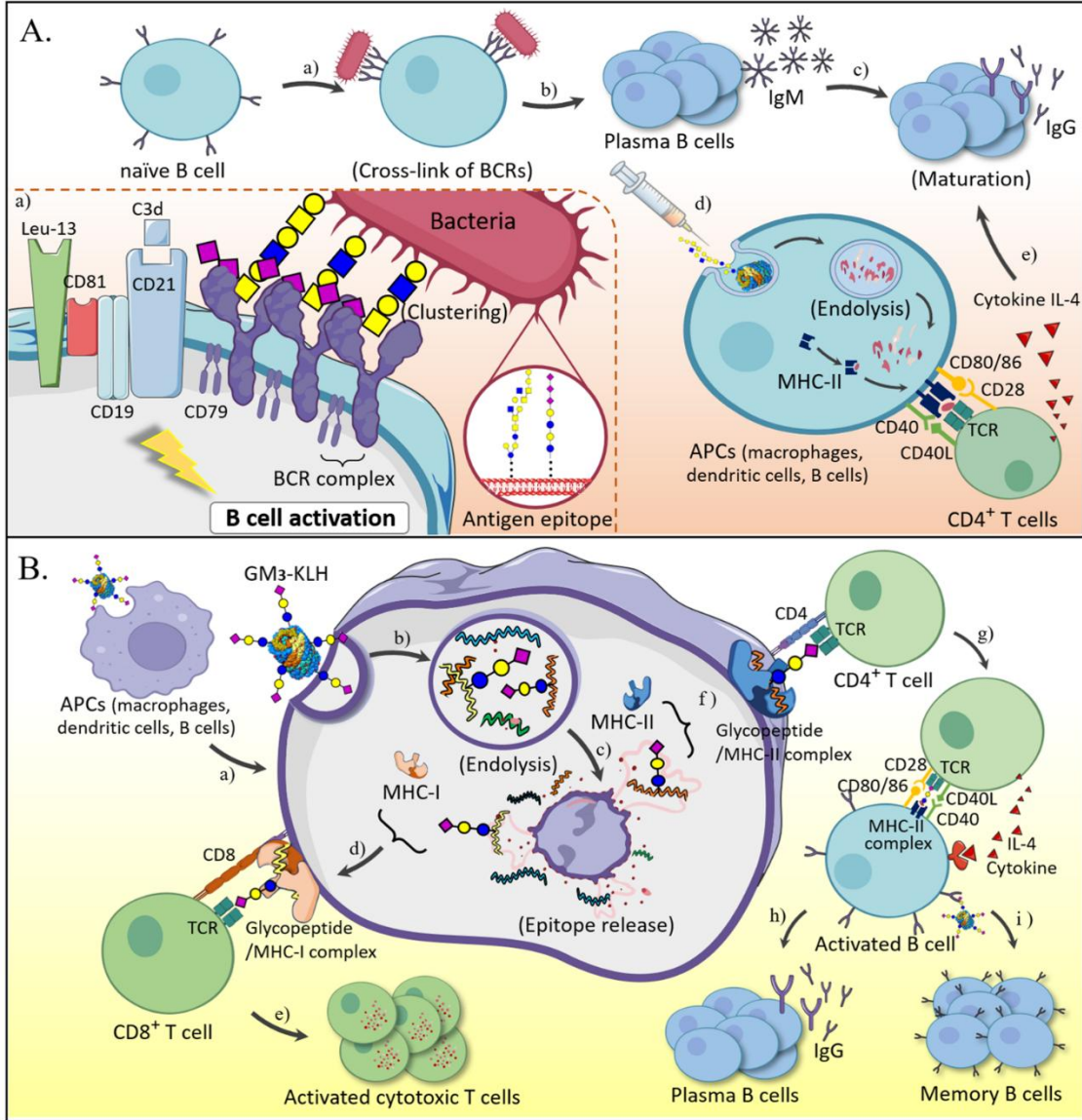
1 intervention. Over the past 30 years, the TACAs have demonstrated potential usefulness in
2 strategies for cancer immunotherapy, including the production of vaccines, mAb therapy and
3 the generation of CAR-T cells [60]. Of them, there are nine TACAs (GD2, GD3, GM3,
4 Fucosyl-GM1, Globo H, poly-sialic acid, Tn, STn, SLe^a) were explicitly included in the
5 tumor-associated antigen list by the National Cancer Institute of the United States [61]. Here,
6 the three major immune pathways of carbohydrate vaccines are compared.

7 **4.1 Polysaccharide vaccines activate B cells by crosslink to the receptor clusters**

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

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

1 immune protection [68]. Furthermore, due to the low or absent expression of CD21 on B cells
 2 in children under 2 years of age, these polysaccharide vaccines often fail to confer early
 3 immunity in newborns [69].



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

1 Glycoconjugate is endocytosed by APCs; (b) Endolysis causes the peptides and glycopeptides; (c) The epitopes are
2 released and face different fates depending on peptide length; (d) Shorter peptide (8-10 residues) is captured by
3 MHC-I and transferred to the surface recruiting CD8⁺ T cells; (e) Recognizing the presented GM3 antigen with the
4 T cell receptor (TCR), cytotoxic T cells are activated and matured; (f) Longer peptide (10-25 residues) is captured
5 by MHC-II and transferred to the surface recruiting CD4⁺ T cells; (g) The activated CD4⁺ helper T cell recognize
6 the B cell which endocytosed and presented the GM3 antigen. With activation of co-stimulatory molecules (CD28
7 and CD40L) by the corresponding ligation, the helper T cell releases cytokines to boost the activation of B cell; (h)
8 The activated B cells further differentiate to plasma B cells and produce IgG, (i) or differentiate to memory B cells.

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

31 **4.2 Glycoconjugate vaccines activate the helper T cells by MHC presenting**

1 Given that glycans can be recognized by the immune system and immunoglobulins,
2 targeting TACAs on tumor cells represents a promising avenue for the development of cancer
3 immunotherapy. Compared to the bacterial polysaccharide antigens, TACAs have a lower
4 degree of polymerization, consisting of oligosaccharides composed of 2~8 monosaccharide
5 units [73, 74]. They cannot directly trigger the B cell immune pathway because the size of
6 TACA molecules is not enough to cross-link to BCRs and make the BCRs come into cluster
7 [66]. After it was proven that glycoconjugates with immunogenic carrier proteins can
8 effectively activate the MHC dependent T cell pathway, making TACA molecules in form of
9 glycoconjugates made it possible to develop carbohydrate based anti-tumor vaccines [75].

10 The strategy to utilize TACA based glycoconjugate vaccines is based on the classic
11 adaptive immune system, also known as the acquired immune system, which relies on the
12 ability to distinguish “self” antigens from pathogen-derived antigens [76]. Take the immune
13 process of GM3-KLH glycoconjugate as an example, this pathway is illustrated as Figure 3B:

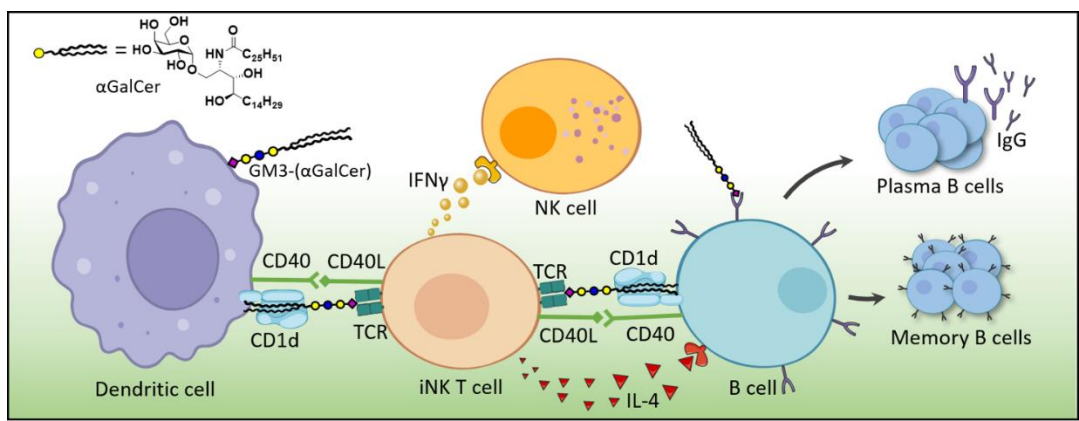
14 a) Upon conjugation an immunogenic carrier protein, the glycoconjugates will be firstly
15 recognized as foreign substances and swallowed by APCs, including macrophages, DCs and
16 B cells [77]. b) During this endocytosis process, the glycoconjugate molecules are surrounded
17 by an area of cell membrane, which then buds off inside the APCs to form a vesicle
18 containing the ingested contents. c) After internalization into APCs, the glycoconjugates are
19 digested by proteases to release peptides, and glycopeptides bringing TACAs. d) Specific
20 8-10 residue long peptides or glycopeptides are then transported to the lumen of endoplasmic
21 reticulum for loading on MHC-I to form a complex [78]. In particular, there are two long
22 α -helices (45 kDa of each) form two walls of a groove-shape like structure. The proper
23 peptides could lie into the groove, and, in the case of glycopeptides, the glycans will extend
24 out [79, 80]. The glycopeptide-MHC-I complex are then transferred onto the cell surface of
25 APCs and recruit CD8⁺ T cells. e) The T cell receptor (TCR) is a disulfide-linked
26 membrane-anchored heterodimeric protein normally consisting of the highly variable α - and
27 β -chains known as $\alpha\beta$ -TCR (95% of all TCRs). The remaining 5% is $\gamma\delta$ -TCR composed of γ -
28 and δ -chain [81]. Studies have shown that $\gamma\delta$ -TCR is related to the recognition of the glycan
29 moiety presented by the glycopeptide-MHC-I complex [82]. Specific TCR interacting with
30 the antigen presented by MHC-I leads to activation and proliferation of the T cells, which

1 further differentiate to mature cytotoxic T cells [83]. These cytotoxic cells will act like the
2 “monitors” to monitor tissues and cells according to the specific antigen information received,
3 e.g. GM3 as example. If cancer cells expose the GM3, they will be eliminated by these
4 cytotoxic T cell by downstream pathways.

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

15 4.3 Glycolipid vaccines activate the iNKT cells by CD1d presenting

16 In recent years, a strategy has emerged that uses glycolipid molecules as immune agonists
17 to present oligosaccharide antigens and generate immune responses. The glycolipids are
18 found to be immunomodulatory molecules which have the ability to activate a unique subset
19 of T lymphocytes, known as invariant natural killer T (iNKT) cells [86]. Glycolipid antigens
20 (example: α -galactosylceramide, α GalCer) are recognized by iNKT cells with the presentation
21 by CD1d, an MHC-I-like molecule (Figure 4). The CD1d molecule is nonpolymorphic,
22 therefore, it allows the certain ligand α GalCer to fit the lipid chain into cavity of CD1d and
23 leave the TACA moiety outside to be recognized by TCR on iNKT cell [87]. After being
24 activated, iNKT cells rapidly release a diverse array of Th1 cytokines (e.g. interferon- γ , IFN γ)
25 and Th2 cytokines (e.g. IL-4) to modulate natural killer (NK) cells and B cells responses,



1 respectively [88, 89]. Besides, the generated cytokines could also induce activation cascades
2 of other immunocompetent cells, such as CD4⁺ T cells, CD8⁺ T cells, DC cells. Thus,
3 α GalCer has been called a “universal helper” due to its powerful self-adjuvant properties [35].
4 **Figure 4.** Pathway of immunomodulation by invariant natural killer T (iNKT) cells. The dendritic cells (DCs)
5 present the antigen GM3 by the α GalCer receptor CD1d on DCs (structure of α GalCer shown in the top left). After
6 the TCR ligation, the iNKT cells are activated and then secrete multiple Th1- and Th2-type cytokines, such as
7 IFN γ and IL-4, influencing the activation of natural killer (NK) cells and B cells, respectively.

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

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

19 **5.1 Exploit more effective carriers**

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

27 **5.1.1 Protein carriers**

28 Covalently conjugating TACAs with immunogenic proteins has been successfully applied
29 to develop anticancer vaccines. Over the years, carrier proteins like KLH, tetanus toxoid

1 (TTox), cross-reactive material 197 (CRM197), bovine serum albumin (BSA), human serum
 2 albumin (HSA) and ovalbumin (OVA) have been extensively utilized in glycoconjugate
 3 vaccines (Table 1). The covalent conjugation of TACAs with these carriers has proven to
 4 significantly enhance the immune response [91-93].

5 Table 1. Common Immunogenic Protein Carriers for TACAs

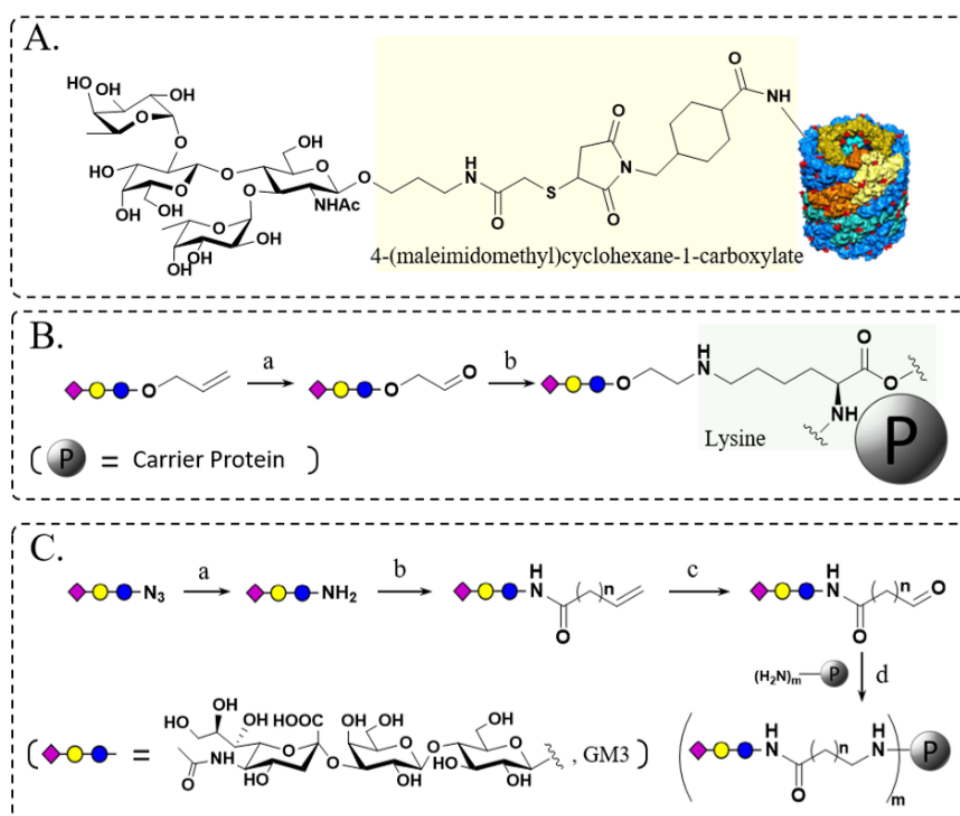
	Molecular Weight	Glycosylation	Multimerization	Amino acid count.	Lysine composition	Database entry
KLH (Keyhole limpet Hemocyanin)	392.2 kDa (S-1) ^a	6 <i>N</i> -sites ^b	decamers/ didecamers of	3414	293 (8.58%)	PDB: 4BED UniProt: Q10583
	391.5 kDa (S-2)	3 <i>N</i> -sites	S-1/2	3421	288 (8.42%)	Q10584
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

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

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

8 In order to make appropriate sugar-protein coupling, the linker holds significant importance.
 9 They increase the efficiency of conjugations by lessening the steric hindrance. Besides, the
 10 proper linkers provide more flexible rotation and enhance the exposure of antigenic epitopes
 11 on the surface of candidates [94]. A number of methods, such as reductive amination,
 12 photocatalyzed thiol-ene reaction, azide-alkyne Click reactions and others, have been devised
 13 to facilitate the glycoconjugation of selected molecular and biomolecular entities [95].
 14 However, some structurally complicated linkers may induce the production of anti-linker
 15 antibodies, which can suppress the IgG antibody response against TACAs. Boons and
 16 colleagues found that a Lewis^y antigen conjugated to KLH using a rigid cyclic
 17 4-(maleimidomethyl) cyclohexane-1-carboxylate linker suppressed the IgG antibody response
 18 against the Lewis^y epitope (Figure 5A). A thorough analysis of sera through enzyme-linked
 19 immunosorbent assay (ELISA) confirmed a robust IgG antibody response going against the
 20 linker region. Therefore, selecting a structurally simple and flexible linker can avoid this

1 problem. A common strategy is to introduce an ethylenic bond onto the reducing end
 2 glycosidic linkage of oligosaccharides, followed by oxidation to form an aldehyde [96].
 3 Subsequently, the oligosaccharide is coupled to the lysine side chain of the carrier protein
 4 through a reductive amination reaction (Figure 5B), and it has been widely applied to the
 5 conjugation of GM3. Zheng et al. loaded 6.6~11.0% and 2.7~5.5% ($W(\text{carbohydrate}) / W(\text{conjugate}) * 100\%$) of GM3 onto KLH and BSA, respectively. The sera obtained from mice
 6 immunized with the KLH-conjugated vaccines showed a significant titer of both IgM and IgG
 7 antibodies against GM3-HSA in ELISA assays. This indicates the occurrence of a
 8 GM3-specific immune response without other signals from the carrier KLH [97]. Guo's team
 9



10 invented a convergent synthesis method for the azido derivative of GM3, along with its
 11 convenient coupling to proteins using an acyl linker (Figure 5C). This coupling strategy was
 12 efficient, with 10.8~13.5% and 11.9~17.5% of carbohydrate loading onto KLH and HSA,
 13 respectively. It was later confirmed that those KLH conjugates of GM3 and three derivatives,
 14 coupled using this method, induced a good GM3-specific antibodies in immunized mice after
 15 35 days [98, 99].

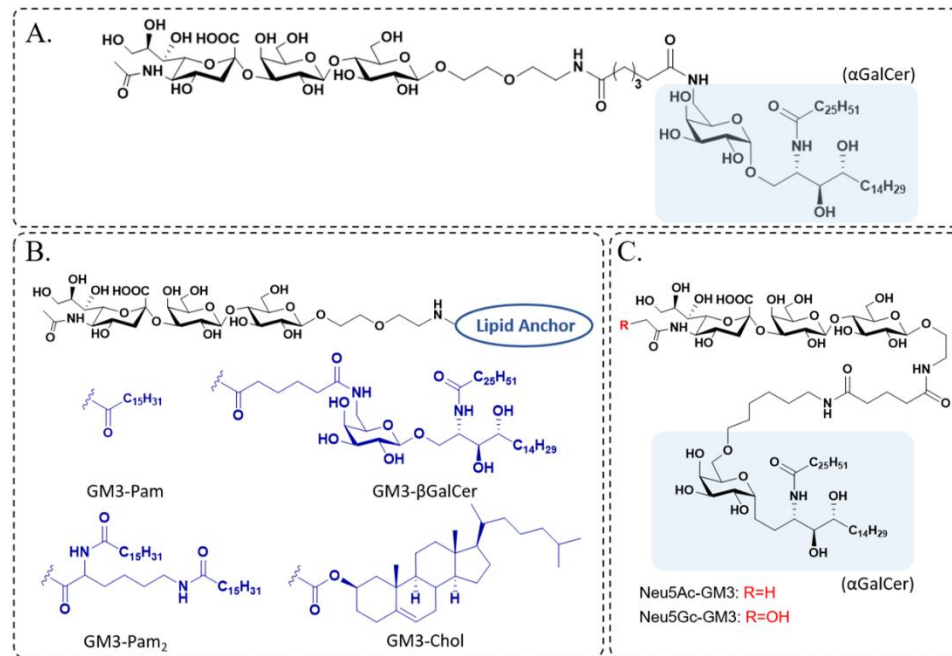
1 **Figure 5.** Representative linkers and general strategies for glycoconjugation to protein carriers. **A.** an
2 example of a rigid cyclic 4-(maleimidomethyl) cyclohexane-1-carboxylate linker illustrating that
3 complex linker structures will compete for immune responses, induce linker-specific antibodies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

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

20 5.1.2 Agonist for iNKT cells

21 As an iNKT cell agonist, αGalCer has been utilized in the development of a self-adjuvant
22 vaccine based on GM3. In a study conducted by Yin et al., GM3 was covalently coupled to
23 αGalCer (Figure 6A). Besides, loaded with 4 different lipid linkers, GM3-lipids
24 non-covalently self-assembled with α-GalCer to form vaccine candidates (Figure 6B). Those
25 chemical synthesized vaccines were assembled with
26 1,2-distearoyl-sn-glycero-3-phosphocholine and cholesterol to form liposomes. With
27 immunization to BALB/c mice, these liposome vaccines were evaluated according to
28 anti-GM3 antibody production as well as the complement dependent cytotoxicity assay (CDC)
29 against the B16F10 cell line. The results demonstrated that, the GM3-αGalCer covalent
30 vaccine candidate can elicit a high level of anti-GM3 IgG production, demonstrating potent
31 binding affinity to cancer cells and effective activation of the complement system. This
32 underlined the importance of covalent linkers in enhancing cancer vaccine efficacy. Although

1 GM3 non-covalently conjugated to α GalCer through four lipid linkers had higher GM3
 2 loading ratios to the liposome vaccines in this study, the results confirmed that these
 3 non-covalently coupled formulations were not as effective as GM3- α GalCer [103].



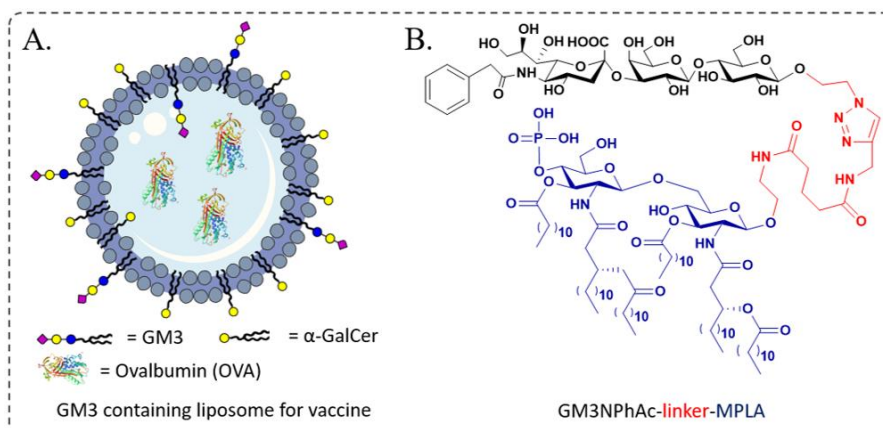
4 **Figure 6.** GM3- α GalCer vaccines, of which the α GalCer (in blue frame) performs the role of iNKT
 5 agonist and also an anchor for composition of liposome vaccine. **A.** A covalent GM3- α GalCer
 6 (self-adjuvant) vaccine candidate; **B.** Four non-covalent GM3-lipid/ α GalCer vaccine candidates.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

10 Romanò et al. reported a straightforward and efficient chemical synthesis method,
 11 covalently coupling Neu5Ac-GM3 and Neu5Gc-GM3 with α GalCer through a linker to form
 12 liposome vaccines (Figure 6C). These uniform liposomes with a controlled size of
 13 approximately 200nm were subjected to *in vitro* and *in vivo* immunological tests on iNKT
 14 cells and C57BL/6 mice, respectively. APCs pulsed with these vaccines were co-cultured with
 15 iNKT cells for 48 hours, a significant increase in the cytokines IL-4 and IFN- γ was observed
 16 in the *in vitro* culture medium. Similarly, murine immunization experiments also
 17 demonstrated that both vaccines could successfully activate the cytokines IL-4, IFN- γ , and
 18 IL-12p70, thereby recruiting Th1 and Th2 T-cell responses. Furthermore, they successfully
 19 activated B-cell responses, resulting in the production of anti-GM3 antibodies. The two sera
 20 induced by Neu5Gc-GM3 and Neu5Ac-GM3 vaccines, respectively, exhibit cross-reactivity

1 against both antigens [104]. This phenomenon is not surprising in TACA vaccine research
2 [105, 106].

3 In addition to coupling the trisaccharide moiety of GM3 to an immunogenic carrier, the
4 complete GM3 ganglioside can also activate immune responses in the form of liposomes.
5 Haan's team reported a GM3 vaccine study based on the iNKT cell pathway. In this study,
6 GM3 ganglioside, α GalCer and OVA were mixed to form liposomes and used for mouse
7 immunization (Figure 7A). Using CD169⁺ macrophage-deficient mice as a control, it was
8 confirmed that this liposomal vaccine could activate downstream iNKT cells through antigen
9 presenting by CD169⁺ macrophages. Furthermore, the liposome immunization group
10 exhibited stronger cytokine production, CD8⁺ T-cell activation and B-cell responses (antibody
11 production) compared to the GM3 ganglioside plus adjuvant group. This study suggests that
12 GM3/ α GalCer liposome can serve as a platform promoting interactions between different
13 immune cell populations, leading to robust adaptive immunity and emerging as a promising
14 anticancer vaccination strategy [107]. Following this, the team reported a more mature GM3
15 antigen-containing liposome vaccine platform: the incorporation of TLR4 or TLR7/8 ligands
16 promotes dendritic cell maturation and phagocytosis, leading to a stronger T-cell response
17 [108].

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



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

4 5.1.3 Pattern recognition receptors (PRRs) as carriers or co-delivery with liposomes

5 Toll-like receptors (TLRs) are a class of transmembrane PRR proteins typically found on
6 sentinel cells like macrophages and DCs. These receptors identify structurally conserved
7 molecules originating from microbes. Once these TLR ligand molecules encounter physical
8 barriers, they are firstly recognized by TLRs, initiating immune cell responses. Consequently,
9 TLR ligands can be employed as carriers for the development of TACA based anticancer
10 vaccines [112].

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

28 With the development of synthetic chemistry, the core structures derived from bacterial
29 LPS have been gradually elucidated and successfully synthesized [118, 119]. The
30 mono-phosphoryl lipid A (MPLA) is 1-*O*-dephosphorylated lipid A, which is a most

1 representative bacterial LPS structure. MPLA exhibits potent immunostimulatory properties
2 and serve as effective vaccine adjuvants. The immunostimulatory activity of MPLAs is
3 ascribed to their capacity to initiate cytokine cascades by interacting with toll-like receptor 4
4 (TLR4). This interaction can trigger antigen presentation, activate T-helper (Th) cells, and
5 consequently induce T cell-mediated immunity [120]. Guo's team identified a highly
6 immunogenic GM3 derivative trisaccharide GM3NPhAc in their former study [99, 121].
7 Based on this, the *N. meningitidis* MPLA was used as vaccine carrier to create a
8 glycoconjugate and administered to mice (Figure 7B). The results revealed that this vaccine
9 elicited an effective immune response to GM3NPhAc without the use of an external adjuvant,
10 particularly leading to the generation of carbohydrate-specific IgG3 subtype antibodies.
11 Interestingly, for one test group in this study was utilized Titermax Gold as adjuvant, but it
12 was observed that the adjuvant did not enhance the antibody production; instead, it appeared
13 to inhibit the GM3NPhAc-specific immune response. This provides insights for selecting
14 carriers and immunization strategies in the development of GM3 anti-tumor vaccines [122].

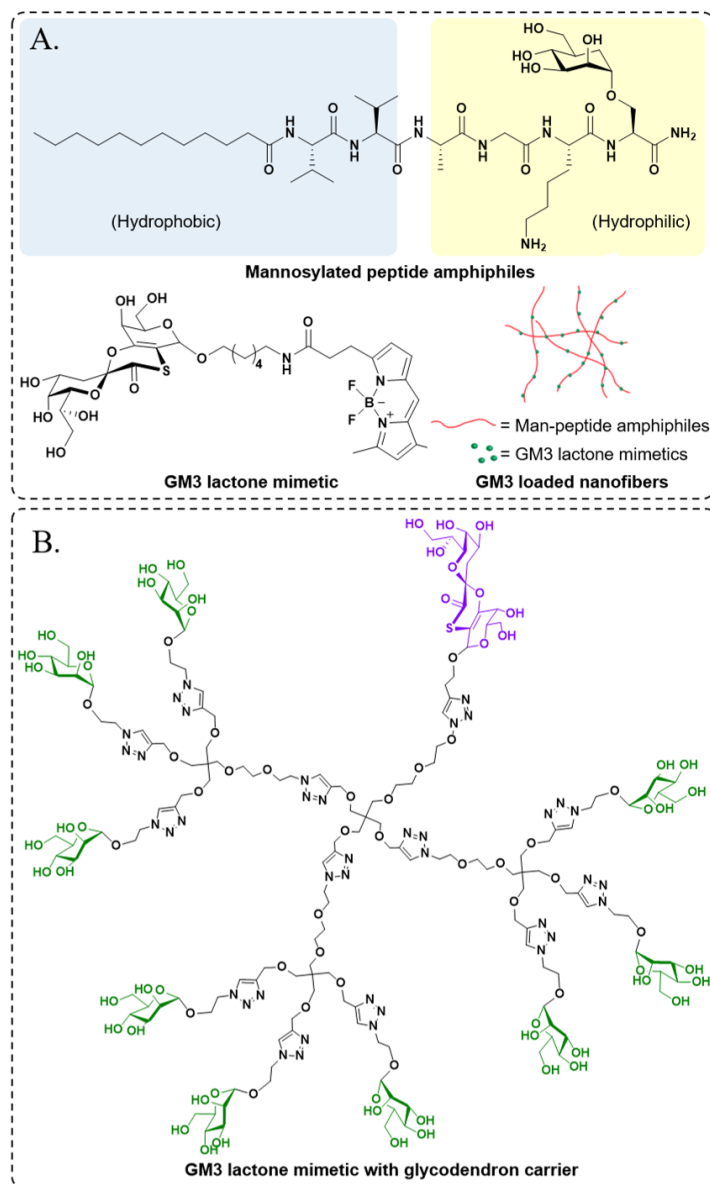
15 In addition to serving as an antigen, GM3 can also be in co-delivered with PRRs by
16 liposomes and facilitate macrophage to capture the liposome vaccines. CD169⁺ splenic
17 macrophages efficiently capture particulate antigens from the blood and transfer them to
18 dendritic cells for downstream immune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

29 5.1.4 Other carriers

1 Other emerging highly immunogenic biomaterials have also been developed as carriers for

1 coupling TACAs to prepare vaccines. For instance, Virus-like particles (VLPs) are complex

1 protein structures that mimic the envelopes or capsids of authentic native viruses but lack the
2 viral genome, falling within a similar particle size range (0.02–0.06 μ m) [124]. Taking
3 advantage of this more natural immunogenic structure, VLPs have been developed for TACA
4 delivery. Huang's group utilized a cowpea mosaic virus (CPMV) capsid as the immunogenic
5 carrier for conjugation with Tn antigen. The resulting Tn-CPMV was administered to mice,



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

11 **Figure 8. A.** Nanofibers loaded with GM3 lactone mimetic as a vaccine candidate. Details

1 seen in Ref. 127; **B.** Structure of a glycodendron consisting of nine mannoses (in green) for
2 DC targeting and one GM3 lactone mimetic (in purple) as antigen. Details seen in Ref. 128.

3 In recent years, with highly regulated dimensions, controllable volumes and abundant
4 surface modification sites, nanomaterials have also been developed as chemical carriers for
5 TACA based vaccines, e.g., gold nanoparticles, nanofibers and other chemical scaffolds.
6 Nativi's group synthesized a thioether-bridged mimetic of GM3 lactone for antigen coupling
7 with various nanomaterial carriers [51, 127-129]. They synthesized a mannose-containing
8 peptide amphiphiles, which can spontaneously assemble into nanofibers in aqueous solution
9 with diameters on the order of 9 ~11 nm and lengths reaching several micrometers. These
10 nanofibers were then mixed with a hydrophobic fluorescent GM3 lactone mimetic, allowing
11 the hydrophobic portion to be inserted into the interior of the nanofiber while exposing the
12 hydrophilic antigenic portion outside (Figure 8A). The abundant mannose on this nanofiber
13 carrier serves as a ligand for DC-SIGN on DCs. By *in vitro* experiments, it was confirmed
14 that these nanofibers could activate DCs to phagocytize the antigens of GM3 lactone mimetic.
15 Furthermore, the over-expression of CD86, CD83 and MHC-II on DCs indicated that these
16 nanofibers could also promoted the maturation of DCs [127]. Similarly, they synthesized a
17 bifunctional multivalent glycodendron consisting of nine mannose residues for DC targeting
18 and one residue of the antigen, GM3 lactone mimetic (Figure 8B). Immunological assays
19 revealed that this glycodendron is able to trigger the activation of human immature DCs and
20 promote the proliferation of T lymphocytes in a mixed lymphocyte reaction [128].

21 **5.2 Chemical modifications on GM3**

22 Protein-carbohydrate interactions present peculiarities compared to general protein-rigid
23 ligand and protein-peptide interactions. Firstly, many carbohydrates exhibit extreme
24 flexibility and contain numerous hydroxyl groups, resulting in the formation of extensive
25 hydrogen-bonding networks that significantly influence the overall binding free energy.
26 Secondly, a notable characteristic of protein-carbohydrate interactions is the formation of
27 essential CH- π contacts between the aromatic side chains of the protein and the C-H bonds of
28 the carbohydrates, particularly on their hydrophobic surfaces [130, 131]. Therefore, the
29 specificity in carbohydrate-antibody interactions is more tolerant. Taking GM3 as an example,

1 numerous studies in recent years have demonstrated that chemically modified GM3
2 derivatives exhibit enhanced immunogenicity during the immune response, and the resulting
3 antibodies show varying degrees of cross-reactivity to natural GM3, which means the
4 antibodies induced by derivatives could fight against the GM3 containing cancer cells [132].
5 This provides an important strategy for the development of GM3 derivatives as anti-tumor
6 vaccines.

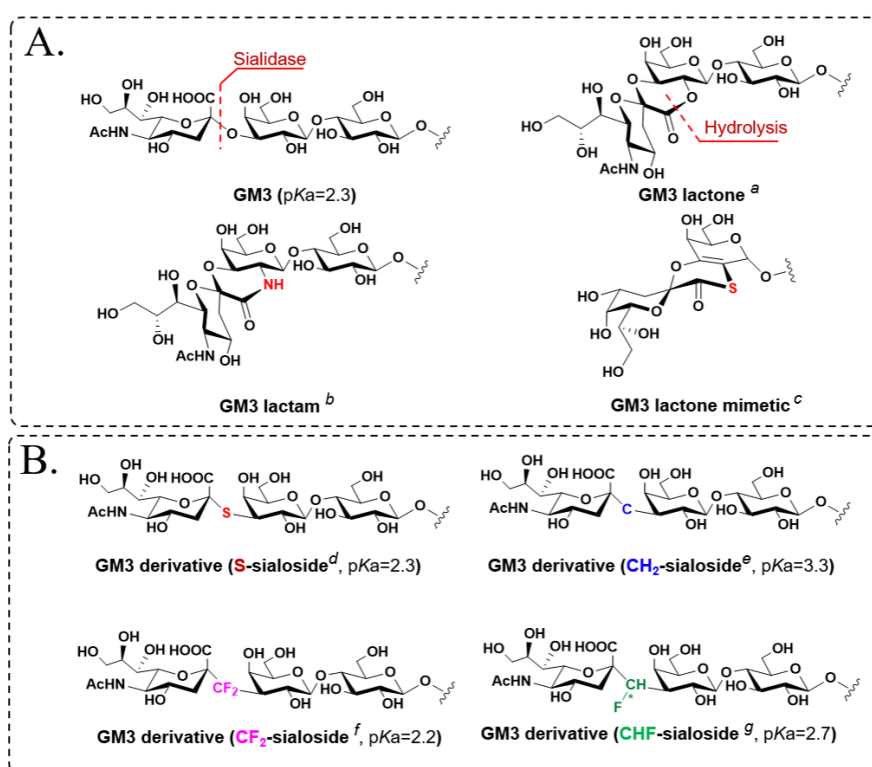
7 5.2.1 GM3 lactone and mimetics

8 As early as 1987, Hakomori's team found that a monoclonal antibody M2590 reacts not
9 only with GM3 but also with GM3 lactone (Figure 9A-a), and the antibody exhibits a
10 significantly higher binding affinity to GM3 lactone compared to GM3. Based on this finding,
11 they immunized BALB/c mice separately with GM3 and GM3 lactone. The results showed
12 that GM3 lactone is more immunogenic, triggering a stronger production of antibodies which
13 were able to bind to both GM3 and GM3 lactone demonstrating cross-reactivity to both
14 antigens [43]. Subsequently, they immunized C57BL/6 mice with GM3 lactone coated on
15 *Salmonella minnesotae*, establishing hybridoma DH2 which secretes IgG3 subtype antibodies.
16 Those antibodies were proven to preferentially react with GM3 lactone over GM3, but
17 inhibited melanoma cell growth *in vivo* and induced antibody-dependent cytotoxicity *in vitro*
18 [133].

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

27 For the same purpose of enhancing hydrolytic stability, Nativi's team synthesized a
28 thioether-bridged mimetic of GM3 lactone (Figure 9A-c) and conjugated it with various
29 carriers for vaccine biological activity testing [135]. In their work published in 2010, this

1 mimetic was conjugated to KLH and used to immunize BALB/c mice. A hybridoma
 2 producing the strongest anti-GM3 antibody was selected, and further analysis showed its
 3 isotypes were identified as IgM and IgG3. Immunocytochemistry results demonstrated that
 4 polyclonal antibodies from the immunized mouse serum bound to HCT116 cells (human
 5 colon tumor cells overexpressing the GM3 antigen) [51]. Subsequently, in 2014 and 2017,
 6 they respectively reported the conjugation of this mimetic of GM3 lactone to non-protein
 7 carriers glycodendron and nanofibers as vaccine candidates, successfully activating DC
 8 immune response.



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

12 5.2.2 Modifications on the glycosidic bond

13 Sialidases are enzymes that can cleave sialic acid glycosidic bonds. For example, the
 14 upregulated sialidase NEU3 in many malignant cancers can cleave *O*- α -2,3 glycosidic bonds
 15 and remove sialic acid from GM3. The exogenous GM3 introduced as anticancer medicines
 16 or vaccines needs to avoid degradation by these glycosidases and maintain its intact structure
 17 to exert biological activity. Therefore, many studies have developed representative

1 sialidase-resistant active analogues targeting sialic acid glycosidic bonds.

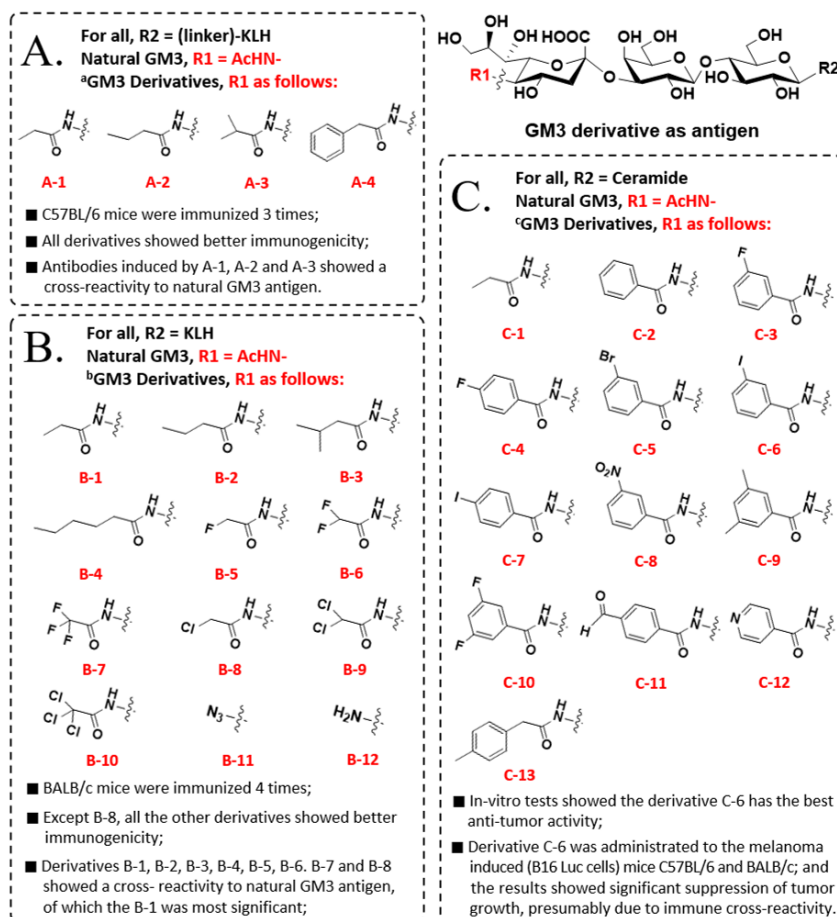
2 One direct method involves substituting the susceptible *O*-sialoside bond in
3 α -2,3-sialylgalactose with an alternative linkage, while minimizing structural alterations to the
4 cyclic sialic acid moiety. Considering this, *S*-Sialosides (Figure 9B-d) was taken into tests,
5 which exhibit greater flexibility compared to *O*-sialosides due to their longer C-S bond,
6 leading to decreased steric and stereo-electronic effects [136]. Bundle's group synthesized this
7 *S*-sialoside substituted GM3 analogue and loaded it onto TTox for immunological assays.
8 Five BALB/c mice were immunized with three doses of *S*-GM3-TTox glycoconjugates on
9 days 1, 21 and 28, and serum antibodies were tested on day 38. The results showed a strong
10 antigen-specific IgG response when immune sera were reacted against *S*-GM3. Additionally,
11 three out of five mice showed a cross-reactivity against natural GM3, with good antibody
12 titers ranging from 10^3 to 10^4 . Analysis of IgG subtypes indicated that this *S*-GM3
13 glycoconjugate vaccine successfully activated the T cell-dependent immune pathway [137,
14 138]. The analysis of molecular structures suggests that while the C-S bond is longer than the
15 C-O bond, the C-S-C angle is significantly smaller than the C-O-C angle. *S*-glycosidic bonds,
16 being less constrained than their *O*-glycoside counterparts, can more readily access the higher
17 energy anti conformation in both unbound and bound states. The replacement of oxygen with
18 sulfur atom leads to longer bonds and weaker stereo-electronic effects, allowing for
19 substantially greater flexibility. This flexibility poses a potential challenge to the use of
20 *S*-glycoside immunogens [139]. According to the *in vivo* experimental results, it appears that
21 this flexibility introduces some randomness but still holds significant inspiring implications.

22 Another category of glycosidic modification for sialidase-resistance of GM3 analogue that
23 has attracted attention is *C*-glycosides. Harai's group has recently incorporated four types of
24 *C*-sialoside linkages (CH_2 -, CF_2 -, (*R*)-CHF-, and (*S*)-CHF-sialosides, shown in Figure 9B-e, f
25 and g) into the synthesis of GM3 using a series of strategies [140]. They first assessed the
26 impact of each glycoside on the pKa value of sialic acid in these *C*-sialosidic analogues. The
27 results showed that the CH_2 -sialoside could increase the pKa of sialic acid (from 2.3 to 3.3,
28 theoretically). Furthermore, in the formation of the *O*- α 2,3 linkage, the lone pairs electrons on
29 the oxygen atom can achieve a more stable conformation based on the exo-anomeric effect. In
30 contrast, the CH_2 -sialoside lacks stereo-electronic effects, resulting in greater conformational

1 flexibility. Therefore, they considered introducing fluorine atoms to maintain the pKa of sialic acid
 2 acid while obtaining relatively stable conformation of the GM3 analogues through the gauche
 3 effect. *In vitro* experiments confirmed that these fluorinated C-glycosidic GM3 analogues
 4 exhibited biological activity similar to natural GM3 by inhibiting the epidermal growth factor
 5 receptor. Additionally, the (*S*)-CHF-linked GM3 analogue with a stable exo-gauche
 6 conformation showed the strongest activity in promoting Had-1 cell growth, indicating the
 7 importance of the specific conformation of GM3 for the biological functions [136]. These
 8 findings suggest promising avenues for research into the immunological activity of such
 9 C-glycosidic GM3 analogues in anticancer vaccine development.

10 5.2.3 Modifications on sialic acid of GM3

11 Taking advantage of the potential cross-reactivity in antibody-carbohydrate reactions,
 12 researchers have introduced chemical modifications to sialic acid in order to disrupt the
 13 immunotolerance and immunosuppression of GM3. Guo's team replaced the acetamido group
 14 of sialic acid and synthesize four GM3 derivatives A-1~A-4 as shown in Figure 10A. The
 15 immunological properties of the GM3 derivative-KLH glycoconjugates were assessed in
 16 C57BL/6 mice with 3 immunizations at day 1, day 27 and day 35. By analyzing the serum
 17 collected on day 35 using ELISA, the results showed that GM3-KLH only activated an IgM
 18 response, while the other four GM3 derivative-KLH conjugates not only activated an IgM



1 response but also elicited a stronger IgG response. Overall, glycoconjugate A-4 exhibited the
2 highest immunogenicity, followed by A-3, A-2, and A-1. However, only sera from mice
3 immunized with A-1, A-2, and A-3 showed cross-reactivity with natural GM3. These findings
4 suggest that larger structural modifications of sialic acid (e.g. A-4) can improve
5 immunogenicity, but the induced antibodies may not react against natural GM3 due to the
6 significant differences in the antibody binding pockets [99].

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

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

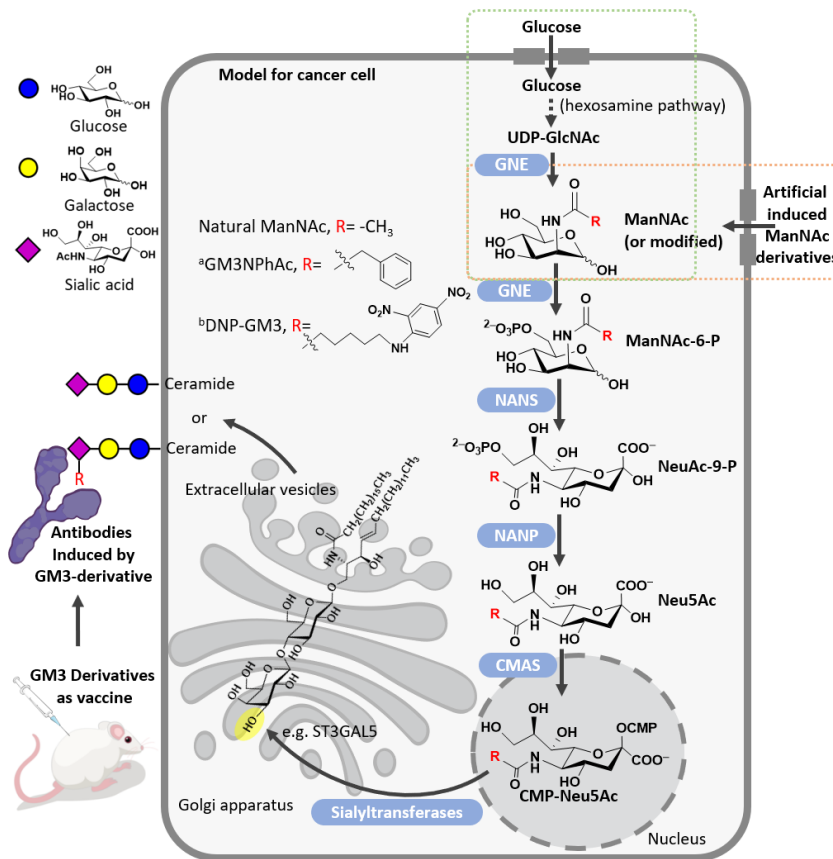
22 In an article published in 2022, Jiang et al. synthesized 13 *N*-acyl modified GM3
23 derivatives (C-1~C-13 shown in Figure 10C) and evaluated their anti-tumor activity by
24 wound healing assays and trans-well assays. Among these derivatives, compound C-6
25 exhibited significant inhibition of cell migration and invasion, as well as marked anti-tumor
26 effects in tumor-bearing (epidermoid carcinoma A431 cells) C57BL/6 mouse model.
27 Subsequent analysis of cancer tissues and serum samples revealed that compound C-6
28 prompted tumor suppression through inducing tumor necrosis and eliciting an inflammatory
29 response, indicating that the tumor inhibition induced by C-6 was closely associated with the
30 carbohydrate cross-reactivity immune response [142]. Although this hypothesis is not entirely

1 consistent with the previous findings conducted by Guo regarding glycoconjugate A-4
2 (GM3NPhAc-KLH), where the introduction of a large phenyl structure to *N*-acyl moiety of
3 GM3 did not elicit cross-reactivity against natural GM3, the promising anti-tumor activity
4 exhibited by compound C-6 still warrants further investigation into immunological pathways.

5 5.2.4 Vaccines based on metabolic glycoengineering of GM3

1 While antibodies generated by GM3 derivatives with more complex modifications (e.g.

1 *N*-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
 4 provide 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
 11 bioorthogonal chemistry, inspired by the groundbreaking work of Bertozzi and colleagues,
 12 and led to Bertozzi being awarded the Nobel Prize in Chemistry in 2022 [146]. Given that this
 13 glycoengineering enables the editing of cancer cell surfaces with modified GM3, the
 14 antibodies activated by the highly immunogenic GM3 derivatives mentioned earlier now have
 15 a role to play. These robust immune responses can selectively target the glycoengineered



1 cancer cells.

2 **Figure 11.** Vaccines based on metabolic glycoengineering of GM3 and an overview of
3 mammalian sialic acid metabolism shown in a cellular sketch. Starting from the top, normally
4 the glucose is taken by cancer cells and converted into ManNAc by following the hexosamine
5 pathway and GNE (pathway details seen in Ref. 144). If a ManNAc with *N*-modification is
6 artificially introduced into the cellular environment, it will participate in the subsequent
7 pathway: catalyzed by enzymes such as GNE, NANS, NANP, and CMAS (pathway details seen
8 in Ref. 143), ultimately transforming into *N*-modified sialic acid. These sialic acids are then
9 added onto GM3 by sialyltransferases such as ST3GAL5, and transported to the cell membrane
10 surface via vesicles. Antibodies and immune responses elicited by immunization with the
11 corresponding GM3 derivative will specifically recognize these glycoengineered cancer cells.
12 Details seen in ^aRef. 147 and ^bRef 149.

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

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

1 response, attributed to the recruitment of anti-DNP antibodies facilitating targeted delivery of
2 the vaccine construct to APCs. The serum obtained through this immunization strategy
3 consisted of IgG subtype antibodies without IgM, and exhibited significant CDC against
4 Man-NHDNP treated B16F10 melanoma cells *in vitro* [149].

5 **6. Conclusion**

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

19 **CRedit authorship contribution statement**

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

24 **Conflict of Interest Statement**

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

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