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O-acetylated gangliosides: Structure, biosynthesis, immunogenicity, functions and their potential for cancer immunotherapy

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Abstract

Sialic acid O-acetylation is a developmentally regulated modification of gangliosides implicated in ontogeny and tumor progression. Their existence has been underestimated in the past because of their alkali-labile nature and their transient expression. New data indicates, however, that O-acetylated gangliosides perform important function in tumor malignancy. Best studied O-acetyl-GD3 blocks the pro-apoptotic activity of GD3 and promotes survival of cancer cells. In acute lymphoblastic leukaemia cells, O-acetyl-GD3 expression level also correlates with survival and drug resistance. The recent identification of the enigmatic O-acetyltransferase opens new experimental approaches for designing novel effective therapeutics targeting drug-resistant cancer cells in acute lymphoblastic leukaemia. In addition, O-acetylated gangliosides expressed at the tumor cell surface are accessible for specific monoclonal antibodies to inhibit cell growth, to induce apoptosis, and to inhibit tumor metastasis formation. Thus, passive immunotherapy using murine or murine/human chimeric monoclonal anti-O-acetylated ganglioside antibodies are currently being investigated. Particularly, targeting of O-acetyl-GD2 could reduce the acute toxicities currently associated with anti-GD2 therapeutic antibodies. This review summarizes the molecular mechanisms involved in the biosynthesis and the expression of O-acetylated gangliosides and presents the new experimental approaches that allow the characterization of their importance in tumor progression. The different strategies used by different teams to develop specific monoclonal antibodies against these poorly immunogenic glycolipids for therapeutic application are also discussed.

Keywords: gangliosides; O-acetylation; O-acetyltransferase; sialic acid; cancer; O-acetyl-GD3; O-acetyl-GD2; apoptosis; antibody; immunotherapy

Introduction

Gangliosides represent an important family of sialic acid-containing glycosphingolipids. They are localized on cell plasma membrane and are amphiphilic, ubiquitous and relatively abundant in the vertebrate nervous system. The hydrophobic region consists of a ceramide that is inserted into the outer layer of the plasma membrane. This ceramide anchor is linked to a hydrophilic glycan chain facing the extracellular milieu, and can interact with other membrane components or other ganglioside-binding molecules such as specific monoclonal antibodies. Based on their higher abundance in tumors when compared with the matched normal tissues, some gangliosides have been suggested for cancer immunotherapy. In this regard, GD2 is the first ganglioside proven to be an effective target antigen for cancer immunotherapy with the regulatory approval of dinutuximab, a chimeric anti-GD2 therapeutic antibody [1]. Interestingly, the use of anti-ganglioside

monoclonal antibodies revealed a larger ganglioside glycan structural diversity, and novel O-acetylated ganglioside species were discovered. Their existence

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might have been underestimated because of their alkali-labile nature and their transient expression during the course of embryogenesis and ontogenesis. In the past years, a better comprehension about their expression and function has identified *O*-acetylated ganglioside species as potential targets for developing new therapeutics to fight cancers. These therapies involve monoclonal therapeutic antibodies to *O*-acetylated ganglioside and targeting specific enzyme involved in their biosynthesis. Here, we report on the historical characterization of *O*-acetylated ganglioside, and discuss recent progresses made in the understanding of their biological function highlighting the importance of these molecules in cancer research.

Structure and nomenclature of *O*-acetylated gangliosides

Gangliosides are acidic-glycosphingolipids that have one or several sialic acid residues in the oligosaccharide chain (Figure 1a). The hydrophobic region consists in a *N*-acylsphingosine, also known as ceramide, which is made up of sphingoid base linked to a fatty acid. The different molecular species of fatty acids forming ceramide vary according to the length of the carbon chain, ranging from 14 to 26 atoms of carbon, but also according the carbon-carbon bond saturation. Thus, the carbon chain can be either, saturated, mono- or poly- unsaturated. The length of the sphingoid base is also variable, ranging from 14 to 20 atoms of carbon. The glycan chain is linked to the ceramide by a β -glycosidic bond. Sialic acids can be attached once, twice, or severalfold to different glycan residues within the oligosaccharide chain. A α 2,3-linkage is involved in the connection between the galactose and the galactosamine. They are bound to other sialic acids by a α 2,8-bond. Ganglioside structural heterogeneity increases when substitutions on sialic acids are taken into account. They can be either *N*-glycolylneuraminic acid (NeuGc) or *N*-acetylneuraminic acid (NeuAc). The latter is the most frequently member found in human while the other is abundant in many other species. The outer sialic acid residue can be further *O*-acetylated [2], but also de-*N*-acetylated, sulfated, or modified by lactonization [3]. Of note, *O*-acetylation of ganglioside terminal sialic acids linked by a α 2,8-linkage to another sialic acid occurs especially during embryogenesis and ontogenesis and are regarded as oncofetal markers present on different type of tumors [4]. The exact position of the alkali-labile *O*-acetylated function on terminal sialic acid stays debated. Depending on authors, ganglioside *O*-acetylation occurs at either C7 [4, 5] or C9 [4, 6] (Figure 1b). These differences can be explained by a spontaneous migration of the *O*-acetyl group from the position C7 to C9 that occurs under physiological conditions [7]. Half-life of isomerization reaction of the free *O*-acetylated sialic acid was estimated around six hours at neutral pH and 37°C [7].

About fifty ganglioside structures have been determined in mammals according to the glycan part, and two nomenclature systems are currently in use to assign names according to the corresponding structure [8, 9]. The oldest nomenclature, initially described by Svennerholm for mammalian cerebral gangliosides in 1964 [8], is defined according to the following rules: gangliosides are

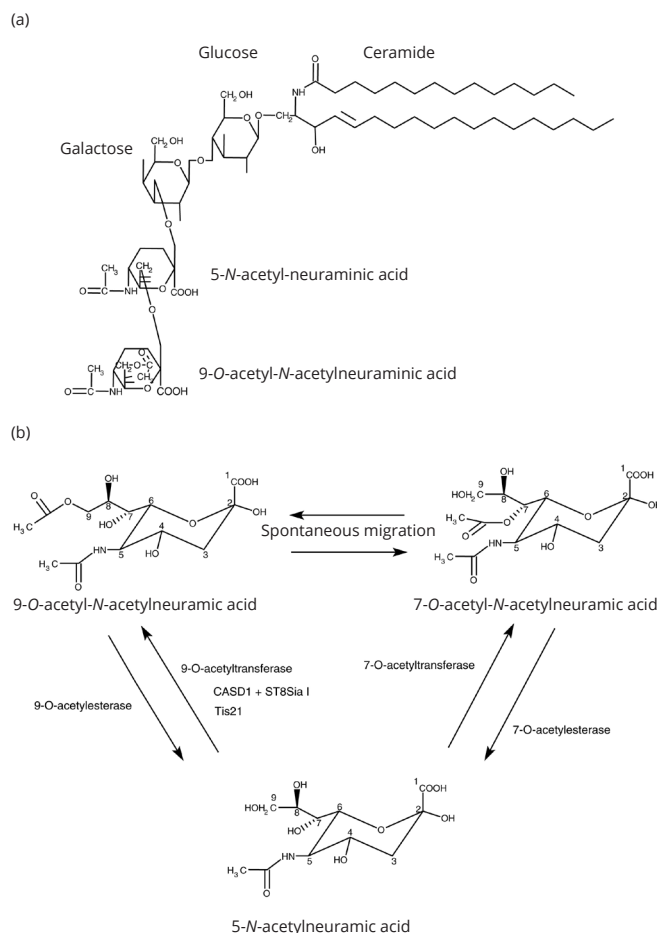


Figure 1 (a) Structure of the disialoganglioside 9-O-acetyl-GD3; (b) chemical and enzymatic relation between the different *O*-acetylated forms of sialic acid found in gangliosides.

identified with the capital letter G whereas the number of sialic acids in the molecule is indicated in subscript by the capital letter M for monosialo, D for disialo, T for trisialo, Q for tetrasialo, and P for pentasialo. These abbreviations are numbered from 1 to 3, and even 4 for monosialogangliosides, corresponding to the migration order of gangliosides assessed by chromatography on thin-layer of silica gels: for instance, GM1 has the lowest migration of monosialogangliosides. A lower case a, b or c indicating the position of sialic acid units within the oligosaccharide chain is also placed after this number. This nomenclature presents the advantage to be convenient and remains the most preferred by researchers. More complex gangliosides have been, however, identified either since and the IUPAC system based on oligosaccharide structure is now recommended by the International Commission on Biochemical Nomenclature [9]. In this system, each ganglioside is named as *N*-acetyl (or *N*-glycolyl) neuraminosyl-(X)-osylceramide, where (X) stands for the designation of the neutral oligosaccharide to which sialic acid is attached [9].

Nevertheless, due to the diversity of sialic acids, these two nomenclatures stay imperfect to precisely name *O*-acetylated gangliosides. For instance, the type of the associated sialic acid is not mentioned. For this reason, an additional nomenclature is generally used [10]. In this way, 9-O-acetyl-*N*-acetylneuraminic acid will be designated by

the abbreviation Neu5,9Ac2, in which acetate groups (Ac) are supposed to substitute the amine group in 5-position and hydroxyl group in 9-position of the neuraminic acid (Neu). In any case, it remains important to be able to appreciate, technically and biologically, modifications of hydroxyl groups of sialic acids on carbons 7 and 9 by addition of *O*-acetyl groups.

Extraction, purification and structural characterization of *O*-acetylated gangliosides

Many methods were suggested to extract and purify gangliosides but none of them is exempt of limitations. Classically, gangliosides are extracted from tissues and body fluids by chloroform-methanol extraction. This extract is then subjected to a Folch partition to separate neutral lipids from acidic lipids [11]. Then, separation of gangliosides according to their glycan head can be achieved by normal phase chromatography using silica gel as stationary phase such as high performance liquid chromatography (HPLC). Purified ganglioside species can be further separated according to their fatty acid composition by reversed phase chromatography [12]. When sample size is limited, a better strategy to achieve purification of ganglioside components is to transfer a ganglioside extract separated on a high performance thin-layer chromatography plate to a PVDF membrane [13]. This simple purification procedure takes advantages of well-established TLC protocols with satisfactory results [13]. All this method can be further coupled to spectrometry analysis for comprehensive structural characterization of ganglioside species.

In all these purification steps, it is essential to protect the original structure of sialic acid because *O*-acetyl groups, and also lactone ganglioside, are lost under alkaline conditions. Many processes used for structural analysis of glycoconjugates will remove *O*-acetyl group from sialic acid. For instance, phospholipid contaminant elimination by alkaline hydrolysis in methanol, structural analyses by peracetylation followed by de-*O*-acetylation, and the release of glycan chains bound to an *O*-glycoside by action of borohydrure followed by an alkaline treatment.

The most practical method to demonstrate the existence of an alkali-labile group on a ganglioside is to start by two-dimensional thin-layer chromatography on silica gel with an alkaline treatment of the plate between two migrations [12]. But, in the case of gangliosides, which have a disialoyl group, nothing permits to discriminate between the formation of inner lactones and the existence of *O*-acetyl group. The use of influenza C virus hemagglutinin esterase enables to distinguish between 9-*O*-acetylated gangliosides and lactone gangliosides [14]. Influenza C virus hemagglutinin esterase specifically recognizes 9-*O*-acetylated sialic acids on which it binds [14]. Of note, 7-*O*-acetylated sialic esters remain resistant to this enzyme [14]. The use of *Cancer antennarius* lectin [15] and achatinin H [16] can also enable to confirm the presence of *O*-acetylated sialic acids. It remains, however, unclear if these two lectins are able to distinguish 7-*O*-acyl from 9-*O*-acyl esters [15, 16]. *O*-acetylated ganglioside species can also be identified by staining on TLC plates with specific monoclonal antibodies (Table 1).

Table 1 Mouse monoclonal antibodies specific for *O*-acetylated gangliosides.

Monoclonal antibodies	Isotype	Antigen specificity	Immunogen	References
D1.1	IgG3	<i>O</i> -acetyl-GD3	Rat neuronal glial B49 cell line	[2]
JONES	IgM	<i>O</i> -acetyl-GD3	Rat retinal epithelium	[33]
UM4D4	IgM	<i>O</i> -acetyl-GD3	Human T lymphocyte ST-1 cell line	[50]
U5	IgM	7- <i>O</i> -acetyl-GD3	Human melanoma gangliosides	[79]
7H2	IgG2a	<i>O</i> -acetyl-GD3	Rainbow trout liver <i>O</i> -acetyl-GD3	[65]
GMR2	IgM	<i>O</i> -acetyl-GD3 <i>O</i> -acetyl LD1	<i>O</i> -acetyl-GD3 absorbed on acidic-treated <i>Samonella minnesota</i> R595	[80]
8B6	IgG3	<i>O</i> -acetyl-GD2	Human neuroblastoma LAN-1 cell line	[36]
60C3	IgG3	<i>O</i> -acetyl-GD2, and GD2	Human neuroblastoma LAN-1 cell line	[36]
A2B5	IgM	<i>O</i> -acetyl-GT3, and GT3	Chicken embryonic retina cell	[35]
Q211	IgM	<i>O</i> -acetyl GT1c, GT1c <i>O</i> -acetyl GQ1c, GQ1c <i>O</i> -acetyl GP1c, and GP1c	Chicken embryonic retinal membrane	[81]

After purification and identification with specific monoclonal antibodies, purified *O*-acetylated gangliosides can be subjected to mass spectrometry analysis for comprehensive structural characterization. Current methods rely mostly on MALDI- and especially electrospray-(ESI) ionization [17]. Most recently, a method to determine the distribution of *O*-acetylated ganglioside in rat brain matrix assisted laser desorption/ionization (MALDI) mass

spectrometry was published [18]. This procedure can be coupled with imaging software to study ganglioside structure and histological distribution [18]. However, this method cannot provide information about the ganglioside three-dimensional structure, in particular of the glycan head. This can be investigated by other means such as nuclear magnetic resonance spectroscopy [19]. In particular, NMR spectroscopy allows the determination

Biosynthesis of O-acetylated gangliosides

Biochemical studies demonstrated that *O*-acetyl group addition occurs in a postsynthetic fashion [23]. Thus, the current proposed model involves a putative *O*-acetyltransferase. This enzyme reaction appears to occur concertedly with sialyltransferases in the Golgi apparatus, and to be acetyl-coenzyme A dependant [23]. Nonetheless, the relevant *O*-acetyltransferase responsible for ganglioside *O*-acetylation has not been isolated or identified; nor have mechanisms for its regulation been elucidated. Direct purification of *O*-acetyltransferase activity has proven difficult and seems to be affected to an inherent sensitivity to membrane solubilization [24]. Heterologous cell-cDNA library pairs were also to the attempted expression cloning of an *O*-acetyltransferase cDNA in COS cells [25-27]. This lead to the identification of several different gene products capable of inducing 9-*O*-acetylation of the outer sialic acid residue of GD3 ganglioside in this cellular model [25-27]. While a first report identified a 47.4kDa gene product similar to the milk fat globule membrane glycoprotein [25], a second study found a 60.9kDa gene product named AT-1 [26]. These two different clones do not have, however, the expected Golgi apparatus localization, and therefore, do not fit with the proposed model established in earlier reports [23]. Later, Shi et al. demonstrated that, in fact, several cDNA products can induce indirect *O*-acetylation in COS cells, some of them can be artifactual [23]. In particular, the

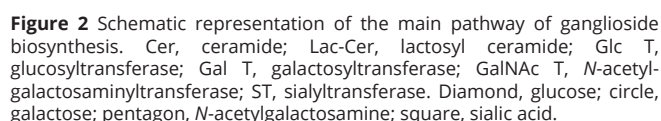


Figure 2 Schematic representation of the main pathway of ganglioside biosynthesis. Cer, ceramide; Lac-Cer, lactosyl ceramide; Glc T, glucosyltransferase; Gal T, galactosyltransferase; GalNAc T, *N*-acetyl-galactosaminyltransferase; ST, sialyltransferase. Diamond, glucose; circle, galactose; pentagon, *N*-acetyl-galactosamine; square, sialic acid.

authors found two clones encoding either a fusion protein between a bacterial tetracycline resistance gene repressor and a sequence reported to be part of expression vector, or an open reading frame identical to the Vitamin D binding protein truncated at the 5' end that eliminates the amino terminal hydrophobic signal sequence [23]. Thus, caution must be taken while attempting to clone this activity using the expression cloning method. More recently, Arming et al. added a new player in the field [28]. In search of a gene candidate that would fit the proposed model of an *O*-acetyltransferase located in the Golgi apparatus, the authors screened the human genome database targeting the genes, which are predicted to possibly encode acetyltransferases and potentially be located in the Golgi membrane. The search resolved the candidate gene CASD1 (capsule structure 1 domain containing 1), which is similar to the CAS1 gene of the fungus *Cryptococcus neoformans* encoding for the Cas 1p protein [28]. The fungal Cas 1p is a membrane protein necessary for the *O*-acetylation of *Cryptococcus neoformans* capsular polysaccharide [29]. Overexpression of the Cas1 protein in combination with GD3 synthase resulted in an increased biosynthesis of *O*-acetylated GD3 while CASD1-specific small interfering RNA reduced *O*-acetylation in tumor cells [28]. Thus, the human Cas1 protein appears to be directly involved in sialic acid *O*-acetylation [28]. In a follow up paper, Baumann et al. gathered stronger evidence of the involvement of

CASD1 in sialic acid *O*-acetylation with a new technological approach [30]. The authors generated CASD1 knockout cells by CRISPR/Cas 9-mediated genome editing. Of note, the authors selected the near haploid human cell line HAP1—derived from the chronic myelogenous leukaemia cell line KBM-7 to generate CASD1 knockout cells. Such a haploid cellular model is often selected in CRISPR/Cas 9 genome engineering, because the genetic variants are easier to obtain than diploid or polyploid cell lines, as only one allele needs to be targeted to obtain a complete loss-of-function phenotype. 9-*O*-Acetylation was completely lost in the resulting CASD1 knockout HAP1 cells, in contrast to the parental HAP1 cells that expressed 9-*O*-acetylated sialoglycans in the Golgi apparatus [30]. In addition, transfection with CASD1 cDNA, but not empty vector, resulted in successful complementation of the loss-of-function defect and restored 9-*O*-acetylation of Golgi-localized sialoglycans in the knockout HAP1 cells [30]. Taken together, these data provide further evidences that CASD1 protein represents a key enzyme in the biosynthesis of *O*-acetylated sialic acids. Yet, unidentified components to cellular sialate *O*-acetyltransferase activities may be required for specific ganglioside *O*-acetylation.

The turnover of *O*-acetyl esters bound to sialic acids of gangliosides is also controlled by sialate-*O*-acetylsterases. Thus, *O*-acetylated gangliosides can either be eliminated during a step of degradation of ganglioside, or participate in a potential acetylation-deacetylation cycle according to the respective level of both sialate *O*-acetyltransferase and sialate *O*-acetylsterase. Several sialate 9-*O*-acetylsterases were discovered and characterized in mammalian cells [31] and in viruses such as influenza C virus [14]. As indicated above, these enzymes bind only 9-*O*-acetyl esters. 7-*O*-Acetyl esters can however migrate at the C9-position and then be hydrolyzed by these enzymes. In mammal cells, two 9-*O*-acetylsterase types seem to exist. The first one is located in the cytoplasm and it is involved in de-9-*O*-acetylation of *O*-acetylated sialic acid delivered from lysosomes [23]. The second one is membrane-bound in the lysosomal compartment [31]. This form contains a cleavable *N*-terminal signal sequence and can be secreted extracellularly and access the 9-*O*-acetylated sialic acids present at the cell surface [31]. However, many details on ganglioside *O*-acetylation and de-*O*-acetylation still require clarification.

Distribution of O-acetylated gangliosides in normal tissues

Gangliosides are found in all cell types, but their concentration and their distribution vary according to tissues and developmental-specific patterns. It is therefore possible to define, for each cell type, a specific ganglioside profile, which is modified during embryogenesis, ontogenesis and tumor transformation [32]. Data collected from *O*-acetyl-GD3 tissue distribution studies suggest that *O*-acetylated ganglioside expression is restricted to proliferating cells and developing tissues. Of note, *O*-acetyl-GD3 was the first identified *O*-acetylated gangliosides using specific monoclonal antibodies D1.1 and JONES [2, 33] and thereby the best studied. It is mainly expressed during rat embryonic development in the central nervous system as well as in the peripheral nervous system, in the retina and

the kidney [33]. Moreover, *O*-acetyl-GD3 was described on human T and B cells as a marker of their proliferation after activation [34]. Other monoclonal antibodies allowed the isolation and the characterization of new *O*-acetylated gangliosides. This is the case of monoclonal antibody A2B5 specific for *O*-acetyl-GT3 [35] or monoclonal antibody 8B6 raised against *O*-acetyl-GD2 [36].

Sialic acid *O*-acetylation is also a developmentally regulated modification of gangliosides, implicated in neural precursor cell migration, peripheral nerve regeneration, and cell proliferation. For instance, rat and mouse fetal cerebral cortexes contain high rates of *O*-acetyl-GD3 and *O*-acetyl-GT3 [37] that will greatly decrease after birth. A comparable evolution was also reported with *O*-acetyl-GT3 in embryonic chicken brain [35]. Kinetics of *O*-acetylated ganglioside expression in embryonic mammal and chicken brains evokes the existence of regulation mechanisms taking part in ovo between day 10 and day 20 [35, 37].

In the central nervous system, monoclonal antibodies specific for *O*-acetylated gangliosides inhibited extension of neurites *in vitro*. More particularly, *O*-acetyl-GD3 ganglioside recognized by JONES monoclonal antibody, may play an important role on the extension of neuronal growth cone and thus on neurite extension [38]. The mechanisms by which *O*-acetyl-GD3 ganglioside expression leads to neuronal migration and neurite outgrowth are still far from being completely understood. Based on the data obtained by others, *O*-acetyl-GD3 may promote these processes by interacting specifically with protein-based adhesion system. An example of interaction between gangliosides and adhesion proteins is the described interaction of GD2 ganglioside with integrin receptor [39]. Although it is tempting to imagine that *O*-acetylation of gangliosides plays a role in the organization of nervous tissue, the obtention of convincing experimental arguments awaits the characterization of the enigmatic specific ganglioside *O*-acetyltransferase. This would allow correlation of phenotypic changes to *O*-acetylated ganglioside expression knockout in a given biological system. Yet, only indirect evidences obtained with specific monoclonal antibodies suggest that these alkali-labile gangliosides are involved in cell differentiation.

Several works also investigated the role of *O*-acetylated gangliosides outside the nervous system. *O*-acetyl-GD3 specific antibodies induce T and B cells proliferation [40, 41], and, in this respect, *O*-acetyl-GD3 was defined as the leukocyte differentiation antigen CDw60 [42]. The use of monoclonal antibodies recognizing the carbohydrate epitopes of non-*O*-acetylated GD3 (CD60a), 7-*O*-acetylated GD3 (CD60c) or 9-*O*-acetylated GD3 (CD60b) revealed a stimulatory or co-stimulatory effect of anti-CD60c and anti-CD60b antibodies on the proliferation of human lymphocytes and implicated distinct roles of 7-*O*- and 9-*O*-acetylated GD3 during activation and apoptosis of tonsillar B and T lymphocytes [40, 41].

O-Acetyl-GD3 is also expressed on suprabasal keratinocytes, which strongly proliferate in psoriasis [43]. In this context, its expression has the particularity to be induced by cytokines, including IL-4 and IL-13, secreted by activated T cells located in cutaneous psoriatic lesions. Noteworthy,

this is the first demonstration that *O*-acetylated ganglioside expression can be induced by a given factor. In addition, these findings could represent a new mechanism by which T cells participate in the pathogenesis of psoriasis.

In order to better understand the significance of the ganglioside sialic acid modifications by an *O*-acetyl group during development and cell proliferation, it is necessary to control endogenous ganglioside expression. On account of the lack of cDNA encoding *O*-acetyltransferases, Varki et al. tried to obtain transgenic mice expressing the influenza C virus hemagglutinin, which is doted of a 9-*O*-acetylerase activity controlled by specific promoters [44]. The expression of the enzyme blocks the development of the zygote from the two-cell stage, suggesting that the *O*-acetylated sialic acid could be involved in the segmentation of the embryo. However, the late expression of the enzyme in some organs creates developmental abnormalities. These observations illustrate, once again, the critical role of the 9-*O*-acetylated sialic acids in cell proliferation and cell migration phenomena.

***O*-acetylated gangliosides in cancer and apoptosis**

The addition of *O*-acetyl group on carbon C9 of sialic acid observed mainly during the embryonic development reappear in some tumors as oncofetal antigens (Table 2). The reported site number in tumors ranges from 10⁶ up to 10⁷ molecules according to the cellular models [45]. For example, *O*-acetyl-GD3, initially described in mammal brain during the embryonic development, is also expressed by melanoma [2], acute lymphoblastic leukaemia [46,47], small cell lung carcinoma [48], glioblastoma [49], and in about half of breast carcinoma [50]. The comparative analysis of the ganglioside profile of hamster melanoma cell variants shows that the predominant expression of *O*-acetyl-GD3 may be closely related to a fast cell growth typical of a low differentiated malignancy grade [51]. Melanocytes also express *O*-acetyl-GD3 when they rapidly proliferate *in vitro* [52]. This expression can be further correlated to other antigenic changes observed in malignancy [51]. At the opposite, absence of expression of *O*-acetyl-GD3 induced by influenza C virus *O*-acetyl-esterase cDNA transfection goes with inhibition of cell proliferation and dendrite formation with a phenotype closed to quiescent melanocyte [53]. Taken all together, these observations suggest that *O*-acetylated gangliosides may promote tumor cell survival and proliferation. The involvement of *O*-acetyl-GD3 in malignancy is also evidenced in acute lymphoblastic leukaemia [47, 54]. The mechanism by which *O*-acetyl-GD3 promote the survival of acute lymphoblastic leukaemia cells involves its anti-apoptotic activity [47, 54]. When Mukherjee et al. added exogenous GD3 to lymphoblast *in vitro*, they induced the apoptotic program in these cells. In contrast, the addition of an equimolar concentration of 9-*O*-acetyl-GD3 failed to induce GD3-mediated apoptosis [47]. It was further evidenced that 9-*O*-acetyl-GD3, unlike GD3 fails to depolarize mitochondrial membranes followed by the release of cytochrome c and activation of caspase-3 [47].

The contribution of *O*-acetyl-GD3 in acute lymphoblastic leukaemia drug resistance was also demonstrated by Parameswaran et al. [46]. They evidenced a strong

Table 2 *O*-acetyl gangliosides expressed in human cancers.

Gangliosides	Cancers	References
<i>O</i> -acetyl-GD3	Melanoma	[2]
	Acute lymphoblastic leukemia	[47]
	Glioblastoma	[49]
	Breast carcinoma	[50]
	Small cell lung carcinoma	[48]
	Basalioma	[82]
<i>O</i> -acetyl GD2	Neuroblastoma	[56]
	Melanoma	[57]
	Glioma	[77]
	Schwannoma	[56]
	Astrocytoma	[56]
	Small cell lung carcinoma	[45]
	Ovarian carcinoma	[83]
	Renal carcinoma	[45]
<i>O</i> -acetyl-GT3	Glioma	[61]
	Breast carcinoma	[62]

induction of *O*-acetyl sialic acid expression, including *O*-acetyl-GD3, in acute leukaemia cells that developed resistance against chemotherapeutic drugs with distinct cytotoxic mechanisms. When they removed intracellular and cell surface-resident 9-*O*-acetyl sialic acid by lentiviral transduction of the influenza C virus esterase, they induced acute leukaemia cell death. In the same condition, the esterase treatment had no effect on normal cells that do not express *O*-acetyl-GD3 [46]. Similar observations were also reported by Birks et al. [49] in biopsy derived human glioblastoma cells, whereby confirming the anti-apoptotic role of *O*-acetyl-GD3. Targeting 9-*O*-acetyl-GD3 expression with sialate-9-*O*-acetylerase resulted in apoptosis in primary glioblastoma cells, with little effect on normal astrocytes [49]. Interestingly, no data are available yet for 7-*O*-acetyl-GD3. Nonetheless, these studies opening new perspectives for gene therapy approaches targeting *O*-acetyl-GD3. However, the influenza C virus hemagglutinin esterase is not an optimal candidate for therapeutic applications because it is strongly immunogenic in patients. Therefore, future developments should exploit inhibition of the sialic acid-specific *O*-acetyltransferases such as the human CASD1 protein [28], or human 9-*O*-acetylerases [55] when these enzymes will be fully characterized.

O-acetyl-GD2 is the *O*-acetyl derivative of the tumor-associated antigen GD2 ganglioside. Expression of GD2 is characteristic of cells of neuroectodermal origin, and high expression level can be found in neuroblastoma [56], melanoma [57] and small cell lung cancer [45], whereas the GD2 distribution in humans is limited to neurons and peripheral nerves [58]. In contrast, *O*-acetyl-GD2 is only found on tumor cells that co-expressing GD2, but not on peripheral nerves and human brain [45]. On neuroblastoma cells, *O*-acetyl-GD2- and GD2 expression levels are similar [45]. Interestingly, *O*-acetyl-GD2 expression level in neuroblastoma cell lines correlates with MYCN amplification that is observed in aggressive neuroblastoma [59]. Based on these observations, *O*-acetyl-GD2 may be involved in malignancy. In contrast, the relation between GD2 expression and tumor cell growth is well documented. Cochonneau et al. reported a possible role of *O*-acetyl-GD2 role in tumor cell death with the monoclonal antibody 8B6 specific for *O*-acetyl-GD2 [60]. The cell death induced by the binding of antibody 8B6 on the target cancer cells was correlated with the expression

of apoptosis-related proteins. The authors demonstrated that *O*-acetyl-GD2 positive tumor cells treated with 8B6 were binding a phycoerythrin-labelled Apo 2.7 antibody when analyzed by flow cytometry. These observations further correlated with a cycle arrest of the cell and increase in p21 protein levels [60]. In addition, expression of apoptosis-associated proteins such as phospho-p38, BAX, cytochrome c in cytoplasm, and cleaved caspase-3 was shown by western blotting in 8B6-treated EL4 cells [60]. The precise mechanisms by which antibodies specific for *O*-acetyl-GD2 trigger tumor cell death remains however unknown. Further ongoing studies should shed light on its functional role in the biological behavior tumors.

Other tumor-associated *O*-acetylated gangliosides such as *O*-acetyl-GT3 and/or *O*-acetyl-GT2 have received less attention [61, 62]. Nonetheless, the above reports suggest that tumor-associated *O*-acetylated gangliosides represent attractive targets for immunotherapy using specific therapeutic antibodies and should warrant further studies highlighting their function in the tumor cell biological behaviour and their therapeutic use in cancer therapy.

Immunogenicity of O-acetylated gangliosides

As described above, *O*-acetyl-GD3, -GD2, and -GT3 are considered as oncofetal marker in human tumors. Thus, there are potential epitopes for antitumor vaccine development [63]. However, raising high-affinity IgG antibody against gangliosides and their *O*-acetylated derivatives remains difficult to achieve because they are weakly immunogenic molecules. The regulation of antibody responses to gangliosides remains furthermore poorly understood to date. Repeated injection of gangliosides alone in human or in mouse is not enough for inducing the synthesis of anti-ganglioside antibodies in vaccinated host. The reason for their poor immunogenicity may be due, in part, to the phylogenetic conservation of ganglioside structures resulting in tolerance. In addition, carbohydrate antigens generally invoke a T cell-independent immune response, during which IgM can be typically driven into IgG3 in naive mice. For this reason, gangliosides are generally classified as T-independent type 2 antigen. Activation of specific B cells to these antigens in the absence of MHC class II-restricted T cell help requires antigen receptor cross-linking which cannot, however, be achieved with small antigen such as ganglioside. Thus, optimization of the immunization protocols against *O*-acetylated gangliosides is required both to generate high-titers of affinity-matured and class-switched antibodies for the production of monoclonal antibodies as tool for research, diagnosis and therapy.

Most of the monoclonal antibodies specific for *O*-acetylated gangliosides generated have been raised by whole-cell immunization (Table 1). In attempts to enhance their immunogenicity in mice, some authors have converted gangliosides to thymodependent antigens by conjugation to proteins or incorporation into protein-enriched liposomes [64, 65]. Successful immunization against purified gangliosides adsorbed on the bacterial cell wall of *Salmonella minnesota* R595 treated in acid condition used as an immune adjuvant was achieved in mice [64]. An adjuvant effect can also be obtained by

incorporating purified ganglioside into heterologous very low-density lipoproteins (VLDL). Using such a protocol, the immunogenicity of *O*-acetyl-GD3 is reinforced in mice and even more when administered with interleukin-2 (IL-2) [65]. A large amount of purified ganglioside is, however, needed to obtain an effective antibody response while using these immunization protocols. This is a concerning factor, given that either purification or chemical synthesis of individual *O*-acetylated ganglioside species remains a challenging task. An alternative approach developed by Katagiri et al. [66] consist of using detergent-insoluble microdomains, or rafts, prepared from specific cell lines as an immunogen. Immunization of mice with such an immunogen induced both an IgM and, to a weaker extent, an IgG response specific for single ganglioside species [66]. These authors did not, however, observed an antibody response against *O*-acetylated gangliosides and it was unclear if the cell lines used in their study expressed *O*-acetylated gangliosides [66]. Whatever immunization protocol used by different authors, the obtained response presents always the characteristics of a primary humoral immune response: (i) the observed isotype is mostly type μ , (ii) the serum antibody titers are low and (iii) these antibodies do not persist a long time in the serum. The booster injections do not lead to a secondary immune response, excluding the formation of memory B cells.

B-cell tolerance to ganglioside antigens evidenced in mice is not absolute. For instance, anti-gangliosides are detected in the serum of patients with cancer when tumors overexpress the ganglioside, suggesting that tolerance can be broken. According to Ravindranath et al. [67], the production of anti-GD3 antibodies in immunized humans with malignant melanoma cells depends on *O*-acetyl-GD3 expression on cancer cells. These data suggest that *O*-acetyl-GD3 is more immunogenic than it's non-*O*-acetylated counterpart and initiate an antibody response in these patients that would bind GD3 by cross reactivity. An interesting phenomenon is that patients suffering from certain tumors such as medulloblastoma display IgM antibodies against *O*-acetyl-GD3 in their serum [68]. The immunogenicity of *O*-acetyl-GD3 in mice and patients depends on the position of the *O*-acetyl group on the outer sialic acid. Ritter et al. [69] observed a specific IgM response against *O*-acetylated GD3 gangliosides only when a mixture of 9-*O*-acetyl-GD3, 7-*O*-acetyl-GD3 and 7,9-di-*O*-acetyl-GD3 was used as immunogen. However, the human and murine immune systems preferentially recognize different epitopes on these molecules. In patients, the immunogen induced antibodies reacted with 7-*O*-acetyl-GD3 form the immunogen and derived from hamster melanoma, but not with 9-*O*-acetyl-GD3 from human melanoma cells. In contrast, all *O*-acetyl-GD3 derivatives used for immunization were recognized by murine antibodies [69].

To bypass potential tolerance to gangliosides in mice, Lunn et al. [70] have used mouse strains knocked-down for GM2/GD2 synthase. These knockout mice do not express complex ganglioside, instead producing predominantly GM3 and GD3 and were used successfully to produce high affinity IgG1 and IgG2b monoclonal antibodies specific for complex gangliosides [70]. Such a model would be useful to obtain high affinity class-switched antibody against complex *O*-acetylated gangliosides.

Another strategy to overcome the marginal immunogenicity of gangliosides, due to their carbohydrate nature and their T cell independence, consists in using surrogate protein structures such as anti-idiotypic antibody [71]. Such mAbs are conceptually easy to generate after immunization of mouse with the parental mAb. However, generating one remains challenging since it requires rigorous selection, as most of the epitopes on the parental mAb will be irrelevant. In resulting mAb, the variable regions form an internal image of the antigen and the mouse constant regions further boost antitumor immune responses in patients [72]. An example of a vaccine development using this strategy is given by racotumomab, a murine anti-idiotypic antibody raised from the anti-de-*N*-glycolyl-GM3 mAb P3 [73]. Racotumomab has been used in several clinical trials and its safety and efficacy were assessed in different tumor localizations: melanoma, breast and lung cancers. More recently, there was a specific interest on pediatric tumors expressing *N*-glycolylated gangliosides. Racotumomab has now reached the phase III clinical trials with possible indications in breast and lung cancer and a possible extent to pediatric tumors [74].

Monoclonal antibody-based immunotherapy targeting O-acetylated gangliosides in cancer

Despite the weak immunogenicity of gangliosides, a limited number of highly specific murine monoclonal antibodies against *O*-acetylated gangliosides were obtained (Table 1). Most of these mouse monoclonal antibodies are generally IgM or IgG3. This is a pity since the IgM isotype is not regarded as ideal for passive immunotherapy. IgG monoclonal antibodies are preferred because they are of higher affinity than IgM antibodies, and their smaller molecular weight allow them to better access the tumor site from the blood circulation. Mouse IgM and IgG3 isotype antibodies also raise some technical difficulties regarding their purification and stability as they tend to clump during their purification and sometimes irreversibly [75]. In addition, most affinities reported are modest with values ranging from 10^{-7} to 10^{-8} M, which may raise specificity issues. Thus, it is advised to establish the antibody specificity in, at least, three systems of analysis to dismiss artifactual conditions. Yet, it is not rare to observe disparities between the different analytical systems [36].

As mentioned earlier, these antibodies have contributed to the identification of novel *O*-acetylated ganglioside species and further suggested their biological functions. Cerato et al. analyzed the structure of the variable VH and VL gene encoding anti-*O*-acetylated gangliosides [36]. They found out that a variety of VH and VL genes encodes for anti-disialoganglioside antibodies. For some of the studied antibody, the VH and VL gene analysis further revealed, surprisingly, the presence of several somatic mutations, suggesting the occurrence of an affinity maturation process [36].

O-acetylated gangliosides present at the cell surface can be targeted by specific monoclonal antibodies (Table 1). Recently, *O*-acetyl-GD2 has received considerable attention as convenient immunotherapeutic target for cancer treatment [45], since it may offer an alternative approach to address the excessive toxicity associated with anti-GD2 therapeutic antibodies such as dinutuximab [58].

Animal studies suggest that this acute toxicity is related to the binding of the antibody to GD2 expressed on peripheral nerves and subsequent Fc domain-mediated CDC activity [76]. Alvarez-Rueda et al. [45] investigated the presence of *O*-acetyl-GD2 on normal tissues, including peripheral nerves using mouse monoclonal antibody 8B6, which only bound *O*-acetyl-GD2 and not GD2. When compared to monoclonal antibody 14G2a specific for GD2, 8B6 stained tumor cells but not peripheral nerves, whereas 14G2a reacted with both. It was further demonstrated that 8B6 monoclonal antibody showed antitumor activities that were comparable to those elicited by anti-GD2 14G2a antibody. Tumor cell killing involved the recruitment of Fc-bearing immune effector cells such as NK cell, and complement deposition at the tumor cell surface [45]. In a follow up paper, it was demonstrated that 8B6 inhibited tumor growth *in vitro* and in mouse models, even in the absence of the recruitment of immune effectors such as NK cell and complement, by inducing cell cycle arrest and apoptosis [60]. These properties provide distinct advantage to monoclonal antibody 8B6 for selectively targeting to neuroblastoma *in vivo* and suggest that antibodies specific for *O*-acetyl-GD2 have the potential to be less toxic than anti-GD2 therapeutic antibodies. Recently, Fleurence et al. demonstrated that *O*-acetyl-GD2 is expressed on human glioblastoma [77]. Furthermore, monoclonal antibody 8B6 induced tumor cell death of *O*-acetyl-GD2-expressing glioma cells *in vitro*, at least in part, via the caspase-3-dependent pathway and by inducing immune effector activity, mainly ADCC [77]. *In vivo*, 8B6 inhibited *O*-acetyl-GD2-expressing glioblastoma tumor growth without down-regulating *O*-acetyl-GD2 expression on tumor cells [77]. This suggests that 8B6 can also be investigated for its anti-glioma properties. Murine monoclonal antibodies are, however, highly immunogenic in patients: they induce Human Anti-Mouse Antibody (HAMA) response in patients, thereby reducing clinical efficacy by removing circulating antibodies. Thus, Terme et al. developed a mouse/human IgG1 chimeric version of antibody 8B6 to facilitate clinical development of therapeutic antibodies targeting *O*-acetyl-GD2 and compared its anti-neuroblastoma activity to dinutuximab, a therapeutic anti-GD2 chimeric IgG1 antibody [78]. Chimeric 8B6 antibody is a novel agent that shares the same anti-neuroblastoma attributes as dinutuximab, with the exception of allodynic activity, when tested in cell-based assay and *in vivo* in an animal model [78]. The absence of *O*-acetyl-GD2 expression on nerve fibers and the lack of allodynic properties of anti-GD2 antibodies, which are believed to play a major role in mediating anti-GD2 therapeutic antibodies dose-limiting side effects, provide an important rationale for the clinical application of chimeric 8B6 antibody in patients with *O*-acetyl-GD2 expressing tumors.

Conclusion

The modification of the ganglioside terminal sialic acid by an *O*-acetyl group has the particularity to be unique to certain tissues and to be controlled by precise mechanisms linked to their development. We are, however, still far from fully understanding their biological role, especially in malignant progression. Many molecular unknowns persist, particularly the identification and characterization of the specific *O*-acetyltransferase involved in their

biosynthesis. Nonetheless, the importance of *O*-acetylated gangliosides in tumor cell physiological pathways that affect tumor progression is evident. In acute lymphoblastic leukemia, survival and drug resistance of lymphoblasts critically depend on 9-*O*-acetylation, which was found on both GD3 and sialoglycoproteins. Indeed, the recent characterization of CASD1 gene product as key enzyme for ganglioside *O*-acetylation opens new perspectives for therapeutic concepts targeting CASD1 to overcome drug-resistant cancer cells in acute lymphoblast leukemia, whose survival crucially depend on 9-*O*-acetylation. Furthermore, the identification of tumor-associated *O*-acetylated ganglioside species makes them attractive targets for cancer immunotherapy. Constant efforts are, however, still necessary to design immunization protocols that allow a high-affinity IgG response for *O*-acetylated ganglioside because of their weak immunogenicity. Based on the example of racotumomab, generation of anti-idiotypic mAb represents a very attractive approach to develop ganglioside vaccines. The use of these antibodies modified by molecular engineering represents, however, an important therapeutic approach in certain cancers such as neuroblastoma. In this respect, the absence of *O*-acetyl-GD2 expression on nerve fibers and the lack of allodynic properties of anti-GD2 antibodies, which are believed to play a major role in mediating anti-GD2 therapeutic antibodies dose-limiting side effects, provide an important rationale for the clinical application of immunotherapeutic strategies in patients with *O*-acetyl-GD2 expressing tumors. Thus, immunotherapy with mAb specific for *O*-acetyl-GD2 has the potential to drastically improve the quality of life of patients. Better tolerance shall allow increasing doses, repeating treatments, further prolonging the overall survival of patients, and allow the development of next generation of targeted immunotherapies. Clinical trials of mAbs targeting *O*-acetyl-GD2 are eagerly awaited.

Conflicts of interest

The authors declare no conflicts of interest.

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