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Gangliosides as Siglec ligands

Ronald L. Schnaar[1]

[1]Department of Pharmacology and Molecular Sciences, Department of Neuroscience, Johns Hopkins University School of Medicine, 725 N Wolfe St, Baltimore, MD 21205, USA

Abstract

The structure of a sialoglycan can be translated into to a biological response when it binds to a specific endogenous lectin. Among endogenous sialic acid-binding lectins in humans are those comprising the 15-member Siglec family, most of which are expressed on overlapping sets of immune cells. Endogenous Siglec ligands are sialoglycolipids (gangliosides) and/or sialoglycoproteins, on cell surfaces or in the extracellular milieu, that bind to and initiate signaling by cell surface Siglecs. In the nervous system, where gangliosides are the predominant sialoglycans, Siglec-4 (myelin-associated glycoprotein) on myelinating cells binds to gangliosides GD1a and GT1b on nerve cell axons to ensure stable and productive axon-myelin interactions. In the immune system, Siglec-7 on natural killer cells binds to gangliosides GD3 and GD2 to inhibit immune signaling. Expression of GD3 and GD2 on cancer cells can lead to tumor immune evasion. Siglec-1 (sialoadhesin, CD169) on macrophages binds to gangliosides on tumors and enveloped viruses. This may enhance antigen presentation in some cases, or increase viral distribution in others. Several other Siglecs bind to gangliosides *in vitro*, the biological significance of which has yet to be fully established. Gangliosides, which are found on all human cells and tissues in cell-specific distributions, are functional Siglec ligands with varied roles driving Siglec-mediated signaling.

Keywords

Sialic acid; Myelin-associated glycoprotein; CD33; Sialoadhesin; Natural killer cells; Macrophages

Introduction

Sialic acid, because of its carboxylate, N-acyl group, and glycerol side chain, is particularly well suited for molecular recognition [1]. The history of sialic acid's discovery is intertwined with its role as a ligand for sialic acid binding proteins [2]. Dr. Roland Schauer provided an account of that history in a recent review [3]. At a conference in Cambridge

Ronald L. Schnaar, schnaar@jhu.edu.

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in 1949 Gunnar Blix (Uppsala Sweden), who first described the chemical properties of sialic acid isolated from submaxillary mucin [4], spoke with Alfred Gottschalk (Melbourne Australia) about the chemical properties of small molecules that Gottschalk found were released from natural sources when incubated with intact influenza virus [5]. Blix wrote later "*It struck me immediately that some of the properties Gottschalk mentioned strongly reminded me about those of sialic acid. When I got home I mailed Gottschalk what we had written about sialic acid and told him that we intended to test the matter closer*." The molecule released by influenza virus was indeed sialic acid. The enzyme that released it was the influenza neuraminidase, and sialic acid was established as the cell surface ligand for influenza hemagglutinin [3, 6, 7]. These discoveries raised interest in sialic acids as molecular recognition molecules and are part of the extensive literature on sialic acids as pathogen receptors for viruses, bacteria and protozoa [8]. This raises the question of why sialic acid is maintained in evolution despite being usurped by deadly pathogens. In this chapter we review some endogenous functions of sialoglycans, with a specific focus on gangliosides, with one family of native human sialic acid binding proteins, Siglecs.

Human Siglecs, Siglec ligands and Siglec functions

In the early 1990's Sørge Kelm and Paul Crocker discovered that "*a subgroup of the immunoglobulin superfamily can mediate diverse biological processes through recognition of specific sialylated glycans on cell surfaces*" [9]. At that time, the subgroup included the macrophage surface protein sialoadhesin (Siglec-1), the B-cell surface protein CD22 (Siglec-2), and myelin-associated glycoprotein (MAG, Siglec4), expressed on myelinating cells of the nervous system. The discovery that these three proteins constituted a structurally related family of sialic acid binding proteins was the birth of research on what are now called Siglecs, sialic acid-binding immunoglobulin-like lectins [10], of which there are 15 in humans [11]. Each Siglec is a single-pass transmembrane protein with variable length short intracellular domains and variable numbers of extracellular immunoglobulin (Ig)-like domains. In each Siglec, the outermost Ig-like domain has the highest sequence similarity to other Siglecs, and contains a shallow surface that binds sialic acid via a conserved arginine residue that engages the sialic acid carboxylate in context of the larger glycan on which it is carried (Fig. 1).

The 15 members of the human Siglec family are notable for their variety of sialic acid binding specificities [11]. Different members of the family take advantage of different ways in which sialic acids are presented on larger glycans. For example, Siglec-1 binds selectively to α2–3 linked sialic acids, Siglec-2 is specific for α2–6 linked sialic acids, and Siglec-7 binds preferentially to α2–8 linked sialic acids (along with certain branched α2,6-sialyl structures) [13]. Binding specificity may also extend further down the glycan chain [9] and include additional glycan constituents, such as sulfation [14]. Human Siglec-XII fails to bind sialic acid (thus its designation by roman numeral), and is included in the family based on its sequence similarity and evolutionary correspondence to sialic acid binding orthologs in primates [15].

Siglecs function in molecular recognition and cellular regulation [16]. Of the 15 human Siglecs, 13 are expressed in overlapping sets of immune cells. Nine of these have one or

more immunoreceptor tyrosine-based inhibitory motifs on their intracellular domains that down-regulate immune responses. Three others have basic residues in their transmembrane domains that associate with immune-activating adaptor proteins. A prevailing hypothesis is that Siglecs on the surface of immune cells encounter and bind to specific sialoglycan ligands in their local environment to initiate signaling pathways that down- or up-regulate of the immune response depending on the Siglec(s) engaged. From this perspective, most of the Siglec family evolved to keep immune responses properly tuned. The one Siglec that is not expressed on immune cells is MAG (Siglec-4), which is exclusively expressed on myelinating cells in the nervous system, as will be detailed further below.

Siglec ligands are sialoglycans that engage specific Siglecs. Endogenous human Siglec ligands range from the smallest class, gangliosides (sialylated glycosphingolipids, ~ 1.5–2.5 kDa) to the largest class, mucins (which may exceed 4 MDa) [17]. In each case, a lipid or protein is decorated in the Golgi apparatus with specific Siglec-targeting sialoglycans. The sialoglycan synthetic machinery is regulated in some diseases resulting in altered expression of Siglec ligands [18-20], and is altered in certain congenital disorders of glycosylation resulting in Siglec-related genetic diseases [21].

Among endogenous Siglec ligands are gangliosides, defined as sialylated glycosphingolipids. All human cells and tissues express gangliosides, with quantities and structures that vary among cell types. Each ganglioside has a hydrophobic lipid moiety, ceramide, firmly embedded in the membrane (primarily the outer leaflet of the plasma membrane) and a glycan that typically extends outward from the cell surface. While there are hundreds of unique ganglioside glycans [22], and even more variation in ganglioside structures based on differences in their ceramide lipids [23], 8 glycan structures make up the majority of gangliosides in human tissues (Fig. 2). These range from the trisaccharide ganglioside GM3 common to many non-neuronal tissues to the four major brain gangliosides (GM1, GD1a, GD1b, GT1b) that comprise > 97% of the gangliosides and the majority of sialoglycans in the human brain [24]. Ganglioside biosynthesis is stepwise (Fig. 2), and mutations in two genes exclusive for glycolipid biosynthesis result in rare congenital disorders. In addition, some gangliosides are overexpressed in cancer, such as GD3 and GD2 in melanoma and neuroblastoma respectively [25, 26]. Changes in ganglioside structures and expression levels impact Siglec-ganglioside interactions and result in human pathology.

Siglec-4 (myelin-associated glycoprotein, MAG)

Among the first proteins identified as a member of the Siglec family was the previously well-characterized nervous system protein MAG (myelin-associated glycoprotein) [9]. MAG is the only human Siglec that is not expressed on immune cells, but instead is found on myelinating cells in the central and peripheral nervous systems (oligodendrocytes and Schwann cells respectively) [28]. MAG has 5 extracellular Ig-like domains and an intracellular domain of variable length that contains a site for Fyn tyrosine kinase phosphorylation. It was implicated as a sialic acid binding protein based on its sequence similarly to sialoadhesin and CD22 [9]. This was confirmed by demonstrating that an expressed soluble tagged form MAG bound to human erythrocytes in a sialidase-sensitive

manner. Erythrocyte re-sialylation using sialyltransferases with different specificities revealed that MAG failed to bind to α2–6 linked sialic acids and had a strong preference for the "3-O" sialoglycan structure: Neu5Acα2-3Galβ1-3GalNAc. Gangliosides are quantitatively the major sialoglycans in the brain [24], and some carry the "3-O" structure (Fig. 2). Subsequent studies confirmed that MAG binds to gangliosides in a physiologically and pathologically relevant interaction revealed by biochemistry [29], mouse genetics [30], and human congenital disorders of glycosylation [21].

Gangliosides are the major sialoglycans in the human brain, carrying over 75% of the total brain sialic acid [24]. Half of that sialic acid is found on two gangliosides, GD1a and GT1b, that carry the "3-O" terminus (Neu5Acα2-3Galβ1-3GalNAc). When gangliosides were stably adsorbed to microwells, they supported binding of cells engineered to express MAG on their surface (Fig. 3). As predicted, GD1a and GT1b supported robust MAG-mediated binding, whereas the gangliosides GM1 and GD1b, which lack the "3-O" terminus, did not. To test the functional roles of MAG-ganglioside binding *in vivo*, genetically altered mice were used. Mice with a disrupted *B4galnt1* gene (Fig. 2) lack the "3-O" terminus specifically on gangliosides. These mice lack all of the major brain gangliosides and instead express comparable concentrations of the truncated gangliosides behind the biosynthetic block, GM3 and GD3 [31, 32]. *B4galnt1*-null mice demonstrated the pathologies of *Mag*-null mice when compared side-by-side, including central and peripheral nervous system axon degeneration that resulted in loss of sensory and motor signal integrity leading to progressive motor behavioral deficits and hindlimb paralysis [30]. Subsequently, similar pathology was discovered in rare human congenital disorders targeting the *B4GALNT1* gene and the *MAG* gene [21]. Mutations in the *B4GALNT1* gene result in complex hereditary spastic paraplegia (SPG26) in several dozen individuals associated with 12 family pedigrees with 12 different *B4GALNT1* gene mutations. The disorder is characterized by weakness and spasticity of the lower limbs with onset typically in childhood and progressing to spastic gait, dysreflexia, muscle atrophy, and speech deficits. Nerve histology revealed axonal neuropathy. In another study, a very rare gene mutation was identified in siblings suffering from a progressive gait disorder and cognitive impairment who were diagnosed with progressive axonal sensorimotor polyneuropathy [33]. The mutation was a single amino acid substitution, R118H, in MAG. That arginine is the very same residue that engages the terminal sialic acid carboxylate of gangliosides. Together, these data support the hypothesis that the "3-O" terminus of gangliosides GD1a and GT1b engage MAG (Siglec-4) to support axon-myelin interactions essential to long term axonal survival. Congenital loss of either the sialoglycan terminus of gangliosides or the amino acid on MAG that binds them results in axonal loss, progressive motor behavioral deterioration and intellectual disability.

The molecular basis for the functional interaction of MAG with gangliosides GD1a and GT1b has been solved (Fig. 4) [34]. The outermost N-terminal Ig-like domain has a shallow surface that binds the terminal Neu5Ac of the "3-O" sequence via a salt bridge to a conserved arginine (R118). When the elongated 5 Ig-like domain structure binds to ganglioside and dimerizes, it decreases the distance between the axon and myelin membranes and facilitates axon-myelin signaling.

Siglec-7

Siglec-7 is expressed by human natural killer (NK) cells, and subsets of myeloid and dendritic cells [35]. It is an immune checkpoint (inhibitory) receptor on cells that functions in tumor surveillance. If sialoglycans expressed on the surface of cancer cells engage Siglec-7 on NK cells, the cancer cells may avoid immune surveillance and replicate. This concept is supported by the consistent finding of enhanced sialylation in many cancers, evidence that has led to the search for Siglec-7 ligands on human cancer cells [36, 37].

Using synthetic glycan arrays, Siglec-7 binds to disialoglycans, including the α2–8 linked disialic acid moieties on gangliosides GD3, GD2, GD1b and GT1b (Fig. 2) [38]. Gangliosides GD3 and GD2 are robustly overexpressed on certain cancers [26] and are target epitopes for melanoma and neuroblastoma immunotherapies [39, 40]. Evidence that cancer cell disialogangliosides engage Siglec-7 to inhibit immune responses was obtained using human neuroblastoma cells [41]. In a multidrug protocol, anti-GD2 antibody sensitized human cancer cells to macrophage-mediated phagocytosis by blocking Siglec-7 binding.

Structural studies revealed the sites on Siglec7 responsible for enhanced binding [12, 13, 42]. In one study [12], the structure of the outermost Siglec-7 Ig-like domain bound to a synthetic analog of GT1b was reported (Fig. 1). The bound disialo moiety and core ganglioside disaccharide (Galβ1-4Glc) were well defined in the crystal such that the terminal sialic acid engaged the conserved arginine residue of Siglecs (R124) with the core disaccharide in a sharply bent configuration relative to the disialo moiety. Binding of the ganglioside mimetic resulted in a large conformational shift that opened up a large hydrophobic patch. It is inviting to speculate that glycan binding, notably of the Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc of gangliosides GD3, GD2, GD1b and GT1b, then enhances engagement with the hydrophobic ceramide. This speculation is supported by a recent study that found that cell surface GD3 containing normal ceramide (see Fig. 2) supports Siglec-7 binding, whereas GD3 containing a ceramide with an extra hydroxyl group on sphingosine C4 (phytoceramide) or a 2-hydroxl group on the fatty acid amide failed to support functional Siglec-7 engagement [43]. These data imply that adding a hydrophilic hydroxyl group near the top of the ceramide may alter the way Siglec-7 interacts with disialo-bearing gangliosides.

Siglec-1 (sialoadhesin)

Sialoadhesin (Siglec-1, CD169) is selectively expressed on human macrophages where it engages self sialoglycans in the extracellular milieu as well as sialoglycans on human pathogens [44, 45]. It does not have immune inhibitory domains, and enhances macrophage phagocytosis of sialoglycan-bearing cargo. Binding to microwell-adsorbed gangliosides revealed that sialoadhesin binds to several gangliosides, with near equivalent binding to GM3, GD1a, GD1b and GT1b [46]. Since pathogenic viruses bud from cells that express some of these gangliosides, binding of viral surface gangliosides to sialoadhesin is implicated in viral uptake by macrophages [45]. This has dual effects depending on the virus and context. Sialoadhesin binding to viral surface gangliosides can result in phagocytosis, degradation, antigen presentation and enhanced viral clearing. Alternatively, gangliosides on

opportunistic viruses may enhance viral entry into macrophages and viral dissemination. Understanding both of these pathways has implications for understanding viral pathogenesis.

Whether helpful or harmful, the above observations led to the use of gangliosides in biotechnology to target nanoparticles to macrophages [47, 48]. A study of nanoparticles decorated with different gangliosides revealed selective binding of GD1a by sialoadhesin-expressing human cells (Fig. 5), and the ability of several gangliosides to enhance nanoparticle internalization [48]. Notably, adding gangliosides to nanoparticles carrying human tumor antigens enhanced the immune response of sialoadhesin-expressing antigen-presenting cells. These findings emphasize that ganglioside binding to Siglecs is a useful cell-targeting biotechnology with therapeutic potential.

Other ganglioside-binding siglecs

Siglec binding to gangliosides adsorbed on microwells was determined for a subset of CD33-related human Siglecs. A summary of the results based on those data are shown in Table 1. While the functional significance of these particular Siglecs binding to gangliosides has yet to be fully explored, it is notable that each tested Siglec had a different binding pattern. As expected, Siglec-7 bound to gangliosides having the α2–8 linked disialo group. Siglec-5 bound preferentially to GQ1b, which has α2–8 disialo groups on both the internal and external Gal residues, whereas Siglec-10 bound to GT1b but not the closely related structures GD1a or GQ1b. Siglec-9 bound to most of the gangliosides tested. In a separate set of studies using microwell-adsorbed gangliosides, Siglec-3 and Siglec-9 bound similar gangliosides, whereas Siglec-8 failed to bind any (Fig. 6). The implications of these findings for Siglec function are the subject of ongoing studies [49, 50].

Concluding statement

The 15 human Siglecs, most of which are immune regulatory receptors, have a variety of endogenous ligands that include sialoglycolipids and sialoglycoproteins that have evolved to serve specific functions in the tissues or on the cells where they are expressed [17]. Strong evidence indicates that certain Siglecs, such as Siglec-4 (MAG) and Siglec-7, are engaged by endogenous gangliosides to trigger important physiological and pathophysiological signaling events. For other Siglecs, the roles of gangliosides as ligands in the context of particular cells, tissues, and biological outcomes have yet to be established. Ongoing studies will provide insights to understand the roles of gangliosides in Siglec signaling. Whatever their endogenous functions, the ability to synthesize gangliosides and incorporate them into nanoparticles and even directly into cell membranes make them inviting Siglec targets for biomedical discovery and possibly therapeutics.

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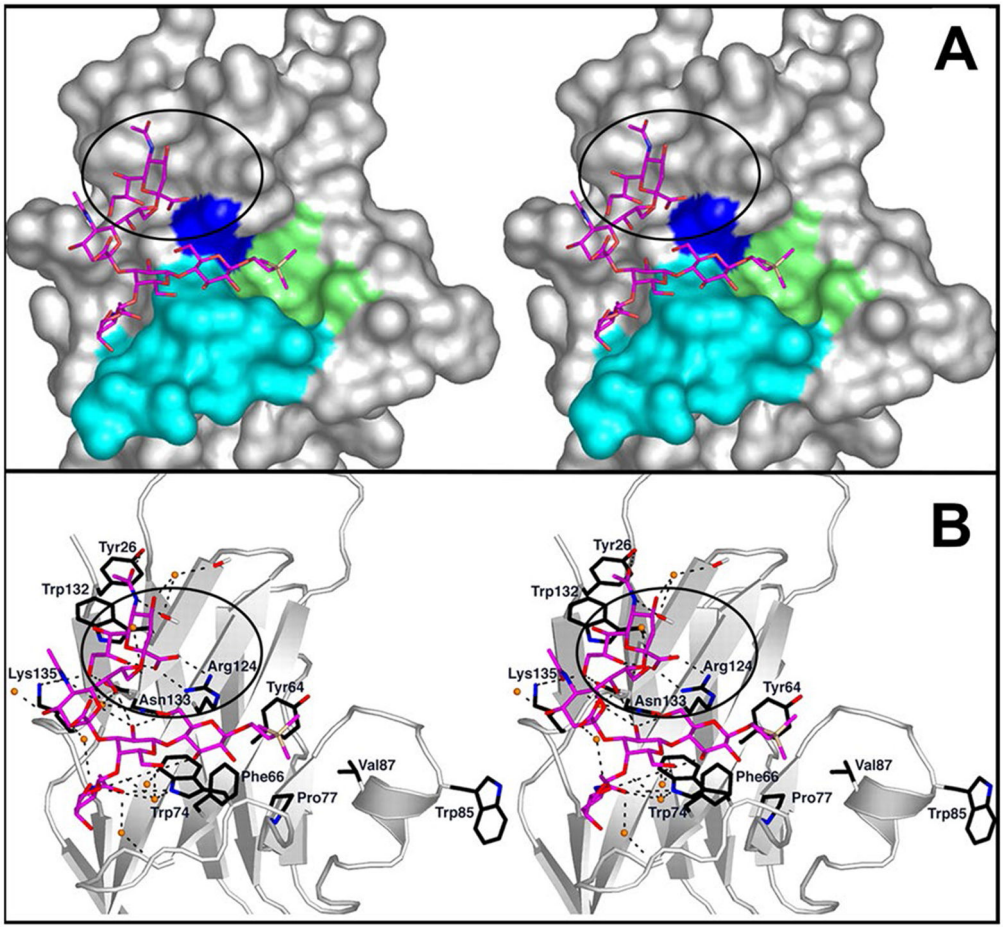

Fig. 1.

Binding of a ganglioside GT1b analog to Siglec7. **A** Stereo image of the outermost Ig-like domain of Siglec-7 bound to a synthetic GT1b analog. Five of the seven sugars of GT1b are resolved at the binding site in the crystal structure (Neu5Acα2-8Neu5Acα2–3[GalNAcβ1-4Galβ1-4Glcβ-R). The terminal sialic acid (Neu5Ac) binds to the conserved Arg residue, R124 (blue). The circled area shows the terminal sialic acid binding via its carboxylate to R124. A convex shelf (marine blue), forms the base of the binding site over which the rest of the glycan lies. The synthetic trimethylsilyl aglycone (yellow) lies in a hydrophobic cup (green). **B** Stereo image of the network of potential hydrogen bonds (black-dashed lines) at the binding site. Stably associated water molecules are shown as orange spheres. Adapted from reference [12]

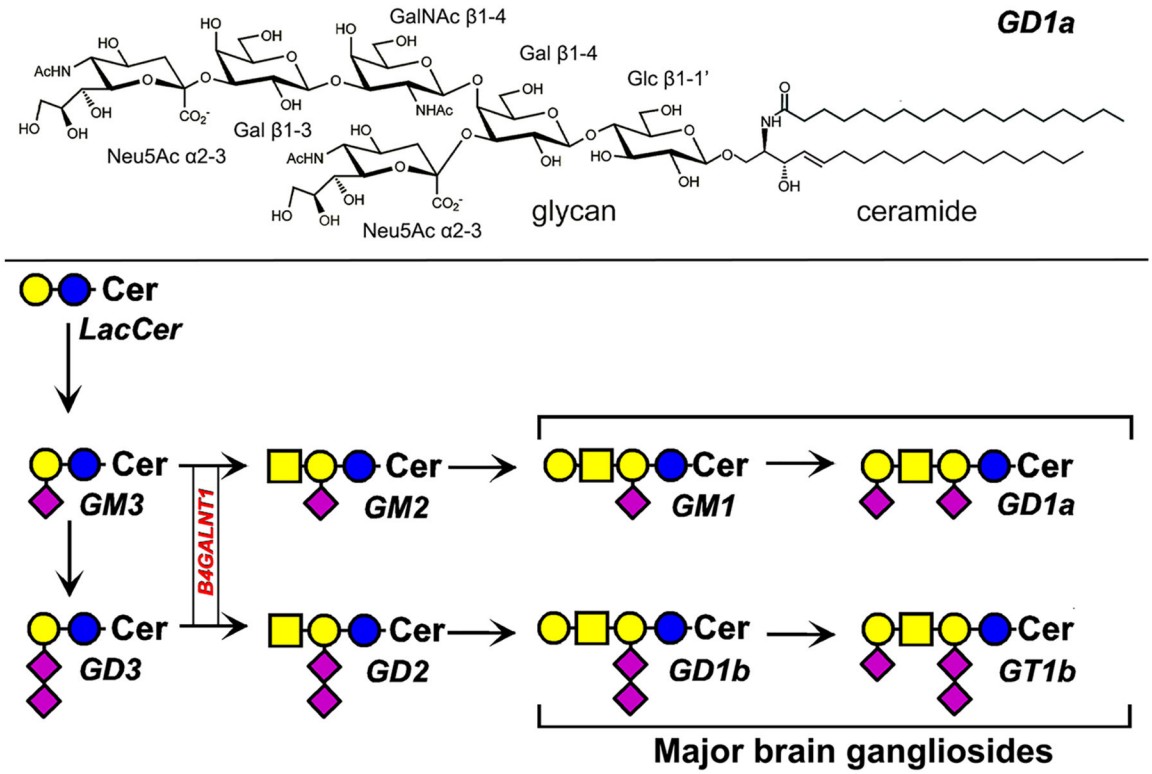

Fig. 2.

Ganglioside structure and biosynthesis. The structure of disialo ganglioside GD1a (top). Biosynthesis of major human gangliosides (bottom) using symbol nomenclature for glycans [27]. The biosynthetic gene *B4GALNT1* discussed in the text is boxed. Cer = ceramide

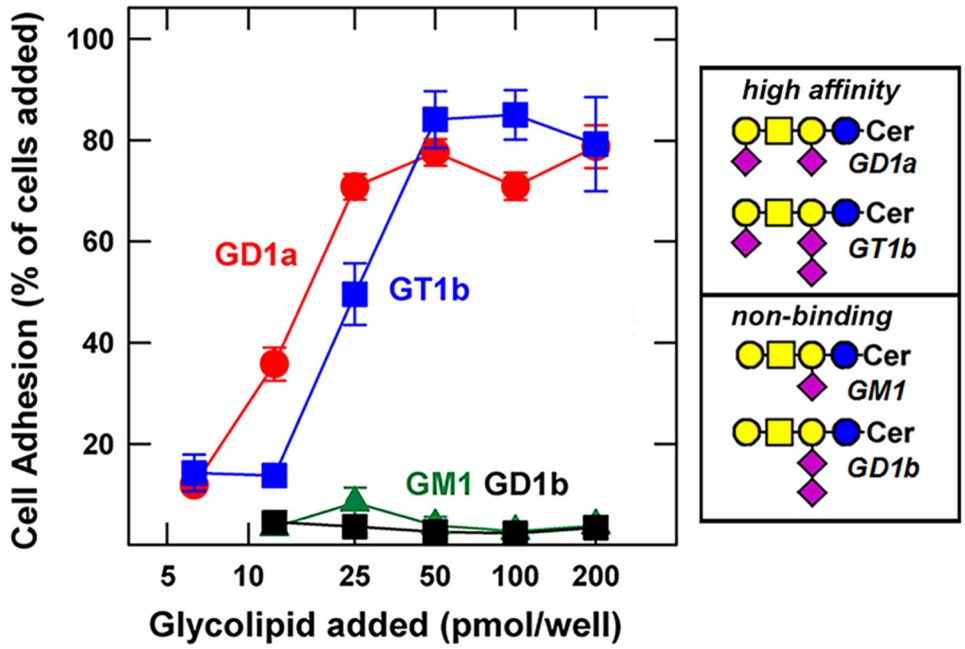

Fig. 3.

MAG binding to major brain gangliosides. Fibroblasts were transfected to express full-length MAG on their surface, then were placed in microwells adsorbed with the indicated concentrations of the indicated gangliosides. MAG-mediated cell adhesion is expressed as a percent of the MAG-transfected cells added to each well. Ganglioside structures are shown using symbol nomenclature for glycans [27]. Image adapted from reference [24]

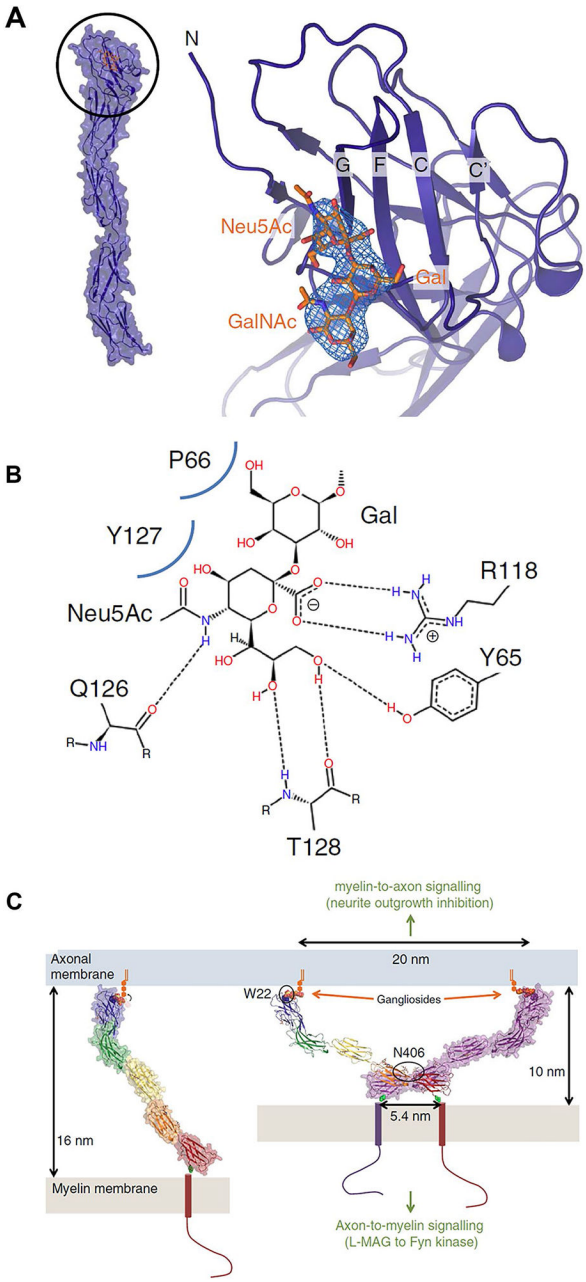

Fig. 4.

MAG-ganglioside binding and a model for myelin-axon engagement. **A** crystal structure of MAG and its terminal Ig-like domain binding to the “3-O” trisaccharide (Neu5Acα2,3Galβ1-3GalNAc, orange). **B** Protein-ligand interactions with hydrogen bonds indicated by dashes and Van der Waals’ contacts by curved blue lines, **C** Model for MAG-mediated myelin-axon engagement and signaling. Dimerization of MAG restricts the distance between the innermost myelin sheath and axon membrane to 10 nm. Reproduced from reference [34]

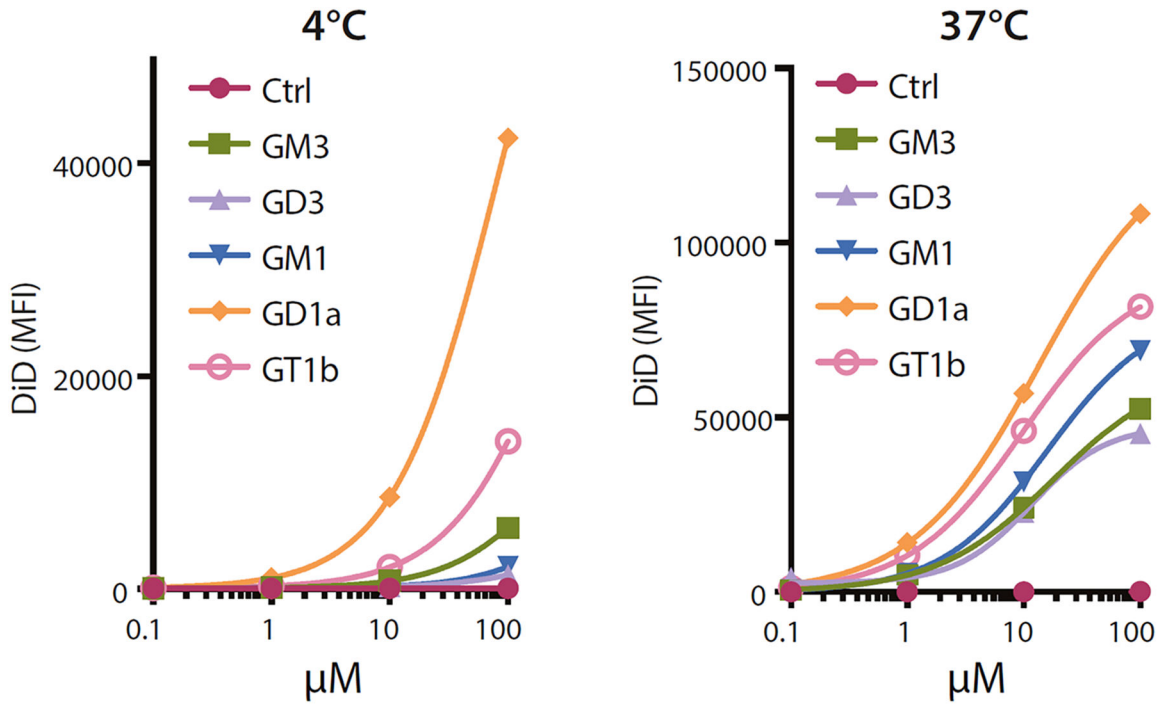

Fig. 5.

Ganglioside-liposomes bind Siglec-1 on THP-1 human monocyte/macrophage cells and are internalized. Fluorescently-labeled ganglioside-liposomes were incubated with THP1 cells overexpressing Siglec-1, and binding at 4 °C or uptake at 37 °C determined by flow cytometry. Binding or uptake of ganglioside-liposomes at different concentrations are shown. Data are from reference [48]

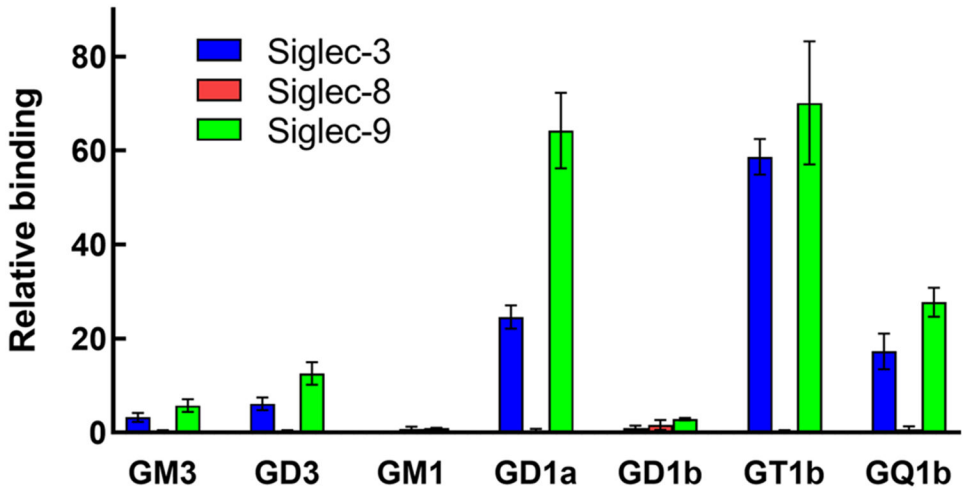

Fig. 6.

Binding of select Siglecs to major gangliosides. Gangliosides were adsorbed to microwell plates and overlaid with soluble expressed Siglec-Fc chimeras precomplexed to alkaline-phosphatase(AP)-labeled anti-Fc antibody. After incubation and washing, bound Siglec was determined by measuring AP colorimetrically. Data are from references [52] and [53]

Table 1

Siglec-ganglioside binding based on reference [51]

Ganglioside	Siglec-5	Siglec-7	Siglec-9	Siglec-10
GM3	−	−	+	−
GM2	−	−	−	−
GD3	−	++	+	−
GD1a	−	−	−	−
GT1b	+/−	+	+	++
GQ1b	+	++	+	−