Glycolipids and CD1: The Crossroad between Chemistry and Immunology

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Introduction

Research during the initial phase of the molecular biology revolution of the 1960s led to the paradigm that biological information, encoded in the genome, flows from DNA, to RNA, to protein. At the time, the understanding of the role of carbohydrates in living organisms was limited, with carbohydrates generally regarded as a source of energy or structural framework within cells and tissues.[1] However, over a decade later, the discovery of carbohydrates that play a vital role in key biological processes such as signalling, cell-cell communication, and molecular recognition opened a new field of research termed glycobiology.[2] A large number of polymers of two or more sugar units (oligosaccharides) decorate the plasma membrane of a cell and are responsible for cellular interactions and the recognition of pathogens. These oligosaccharides are connected to a non-carbohydrate portion, usually a protein or a lipid, giving rise to glycoconjugates known as glycoproteins and glycolipids, respectively.

Given that the immune system operates largely via receptor-ligand recognition, it is not surprising to find that glycoconjugates are capable of activating and modulating the immune response. Indeed, the blood group determinants, first noted by Karl Landsteiner over 100 years ago[3] but not structurally elucidated until the 1960s, are carbohydrate antigens found on the surface of red blood cells.[4] It is these carbohydrates, in the form of glycolipids and glycoproteins, that are responsible for the rejection observed during blood transfusion with incompatible blood groups.

Numerous bacterial cell wall glycolipids have also been found to have an important role in the immune response. In particular, the discovery that mycolic acid [a non-peptide lipid antigen from *Mycobacterium tuberculosis* (*MTb*)] stimulates T cells via a family of proteins called CD1, paved the way towards the discovery of a host of glycolipids that activate the immune system in a similar manner.[5] Subsequently, other glycolipid cell wall components of *MTb*, such as glucose monomycolate (GMM), phosphatidylinositolmannoside (PIM) and lipoarabinomannan (LAM) were found to be immunogenic.[6]

We present here some of the key glycolipids involved in regulating the immune response via their interaction with CD1 and T cells. Particular emphasis is placed on the glycolipid structure, the source of the glycolipid, and the specific type of CD1 protein with which the glycolipid interacts.

CD1 Proteins

CD1 molecules are proteins found on the surface of white blood cells including dendritic cells, macrophages and B cells. Collectively, these cells are known as antigen-presenting cells (APCs). In humans, the CD1 family consists of five members, CD1a-e, that are further classified into three major groups depending on their amino acid sequence. Thus, CD1a-c belong to group 1; CD1d to group 2; and CD1e to group 3. CD1 has a binding groove that is narrow and deep and consists of hydrophobic amino acids that are able to accommodate long alkyl chains of glycolipid tails.[7] In a CD1-glycolipid complex, the polar head group of the glycolipid protrudes from the CD1 binding pocket (Fig. 1).[8] This complex is recognised by T cells via the T cell receptor (TCR), and the interaction of CD1-glycolipid-TCR initiates a cascade of intracellular signalling which activates the T cells to produce signalling molecules, termed *cytokines*. The type of immune response generated is dependent on the cytokine profile produced.

The exact effect that the structure of the glycolipid has on CD1-glycolipid-TCR immune response is only known in a very general sense. It has been proposed that the stability of the glycolipid-CD1 molecule dictates the duration of T cell stimulation, which in turn influences the immune response. A prolonged TCR stimulation is thought to lead to a pro-inflammatory response whereas a transient TCR stimulation gives an immunomodulatory response.[9] Other processes such as CD1 and glycolipid trafficking into the cell, and glycolipid processing have also been found to affect the immune response.[10] Modification of the sugar head groups also affects TCR recognition of the glycolipid-CD1 complex, suggesting that the interaction between the TCR and the glycolipid-CD1 complex is highly specific. The ability to control the immune response via the development of specific CD1-binding glycolipids is a desirable research objective. It can be useful in the treatment of many diseases, including cancer, bacterial infections and autoimmune diseases such as multiple sclerosis and systemic lupus.

A CD1 molecule consists of two chains, the β_2-microglobulin chain and a heavy chain with three extracellular domains (α1-α3) (Fig 2A).11 The heavy α chains form hydrophobic binding pockets, A' and F', that are able to accommodate the lipid tails of glycolipids, as shown by the crystal structure of CD1d bound to the glycolipid α-galactosyl ceramide (Fig. 2B).[12] An extensive hydrogen bonding network holds the sugar head group in place for recognition by the TCR.

In a similar manner to CD1d, the human CD1a molecule possesses the A' and F' binding pockets (Fig. 2B). The human CD1b isotype, however, has four binding pockets, A', F', C' and T', and is thus able to accommodate alkyl chains up to 80 carbons in length.[13] To date, there are no high-resolution structural data for CD1c, but computa-

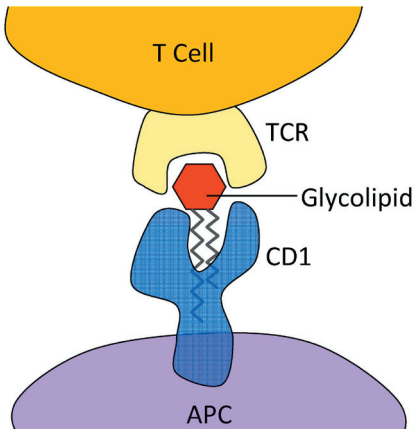


Fig. 1. Representation of CD1-glycolipid-TCR interaction during glycolipid antigen presentation.

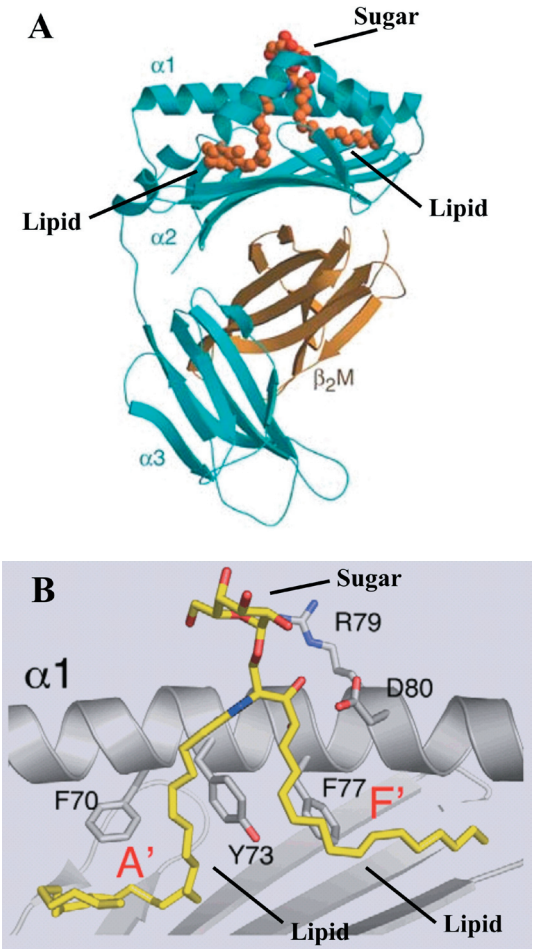


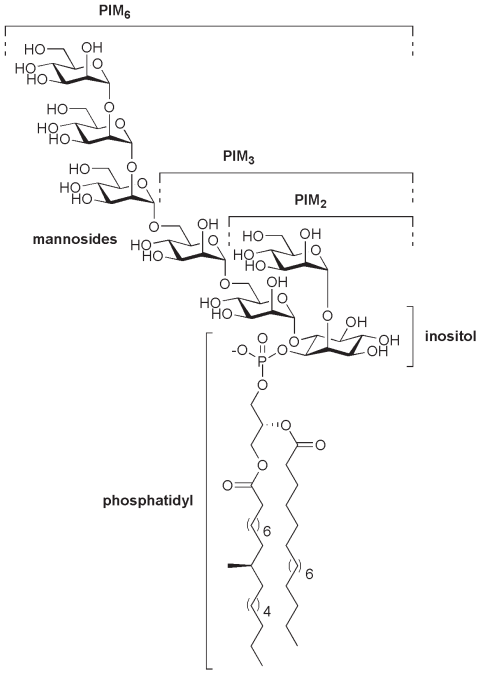
Fig. 2. Human CD1d complex with α-galactosyl ceramide: A) CD1d structure (α1–α3 domains, blue; β_2M, yellow); adapted with permission from Macmillan Publishers Ltd., Nat. Immunol - ref 1], copyright 2005; B) a side view of crystal structure of human CD1d in complex with α-GalCer. The α2-helix is removed for clarity and the side chains of some key amino acids are indicated. Reprinted from ref. 12 with permission; copyright 2007, Elsevier.

tional studies of it in a complex with a *MTb* glycolipid mannosyl-β1-phosphomycoketide (MPM) indicate that the enlarged F' pocket is responsible for binding bacterial polyketide with a multiply branched, unsaturated alkyl tail.[14] CD1e has low homology with other CD1 proteins. It has only a spacious single binding pocket but it can accommodate large lipid molecules. Unlike CD1a-d, which are expressed on the cell surface of antigen-presenting cells, CD1e is found primarily in the Golgi compartment and is believed to be crucial for glycolipid processing. Given the differences in structure of the CD1 molecules, it is not surprising that each shows some degree of specificity with respect to the glycolipids they bind.[8] Roughly, the CD1-binding glycolipids are divided into three classes: the phosphoglycolipids, the mycolates and the sphingoglycolipids. Each of these classes of compounds, and their effects on the immune response, is discussed below.

Phosphoglycolipids

Phosphoglycolipids are glycolipids that contain a hydrophilic polar head group (a sugar moiety) connected to one or more phosphate groups, which in turn, are connected to a hydrophobic tail comprised of two fatty acyl chains. They are commonly found in cell membranes and can form lipid bilayers. Phosphoglycolipids that bind to CD1 and induce T cell responses are phosphatidylinositol mannosides (PIMs; **1**) mannosyl-β1-phosphodolichol MPD; **2**) and mannosyl phosphomycoketides (MPM; **3**).

Phosphatidylinositol mannoside



1a, PIM_2; **1b**, PIM_3; **1c**, PIM_6

Fig. 3. Phosphatidylinositol hexamannosides (PIM_2-PIM_6).

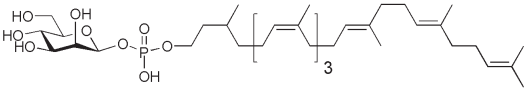
The PIMs **1** were first isolated from *MTb* in 1930,[15] and were characterized in 1960 by Ballou and colleagues.[16] *MTb* contains several PIM structures, including PIM_1, where the inositol residue of phosphatidyl-*myo*-inositol (PI) is mannosylated at the C-2 position, and PIM_2 (**1a**) where PIM_1 is further mannosylated at C-6 position of the inositol moiety. Further α-1,6 mannosylation of PIM_2 gives rise to PIM_3 (**1b**) and PIM_4 – the common precursor of PIM_5 and PIM_6 (**1c**), which are made by consecutive α-1,2 mannosylation of PIM_4.

In 2004, PIMs were shown to be a natural antigen for CD1d-restricted T cells.[17] In this study, a number of mycobacterial lipids were tested, but only a mixture of

different PIMs stimulated NKT cells via CD1d binding, and triggered an antigen-specific IFN-γ production and cell-mediated cytotoxicity. The structural parameters for CD1d binding included the need for two acyl chains on the phospholipid (PIMs may also contain a lipid at the C-6' position of PIM_2, but this is not necessary for CD1d binding), and the presence of a polar head group was required for recognition by T cells. Interestingly, CD1e activates lysosomal mannosidase in order to break down larger PIM analogues, *e.g.* PIM_6, into PIM_2 that can then be identified by CD1d.[18] PIMs have potential to be used as adjuvants, as seen in studies by Painter and co-workers,[19] where it was found that PIM ether analogues activate immature bovine dendritic cells. The first total synthesis of PIM_2 and PIM_6 was reported in 2006,[20] followed shortly thereafter by the synthesis of an alternatively acylated analogue.[19]

Mannosyl-β-1-phosphodolichol

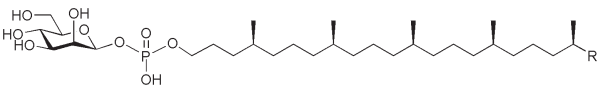
Mannosyl-*β*-1-phosphodolichol (MPD) belongs to the family of glycosyl-1-phosphopolyprenols that are found in all cells. Amongst different organisms, the long chain isoprenoids differ in length, saturation, phosphorylation and glycosylation. MPD consists of a mannosyl *β*-1-phosphate moiety attached to a partially saturated polyprenoid lipid. Multicellular organisms have the longest ($C_{90\text{-}100}$) dolichols, while fungi and protozoa have shorter chain lengths ($C_{70\text{-}90}$ and $C_{50\text{-}65}$, respectively).[21] Synthetic MPD, with shorter chain lengths ($C_{30\text{-}34}$), have been shown to stimulate T cells,[22] while no activation was observed for lipids with longer chains ($C_{55\text{-}95}$). MPDs with a chain length similar to those found in Mycobacteria ($C_{30\text{-}35}$) give the strongest T cell response.



2

Mannosyl-β-1-phosphomycoketide

Mannosyl-*β*-1-phosphomycoketides (MPMs, **3**) are potent mycobacterial antigens, and have been isolated recently from *MTb* (**3a**) and *M. avium* (**3b**).[21] These phospholipids contain a mannosyl-*β*-1-phosphate moiety similar to that found in **2**; however, the lipid portion consists of a pentamethylpentacosyl unit which is fully saturated and was first reported by Crich and Dudkin[23] in 2002, and more recently by Van Summeren and colleagues.[24]



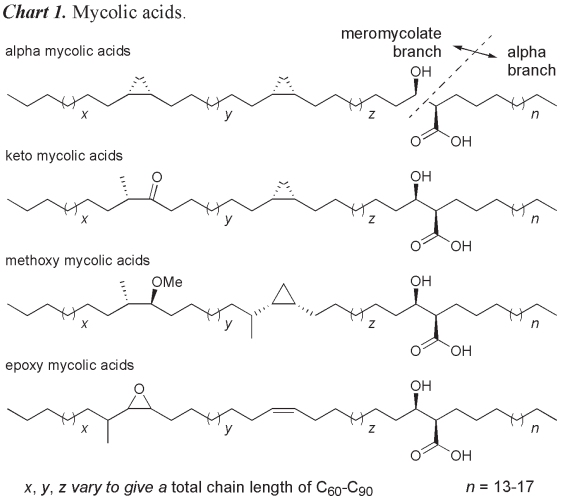
3a, R = heptyl (*M. tuberculosis*)
3b,R = pentyl (*M. avium*)

MPD and MPM are the two classes of antigens that are presented by CD1c molecules. The presence of repeating isoprenoid units in both antigens and their binding to CD1c isoforms indicates that methyl branching is an isoform-specific motif for CD1c-presented antigen.[21] Indeed, in recent work by Jong *et al*.[22] it was shown that methyl branching in MPM and MPD contribute to T cell activation. It is thought that the repeating units may help retain the lipid inside the CD1c groove, in the same way as straight chain lipids interact with the unbranched A′ pocket of the CD1a molecule.[22] Studies of MPM from mycobacterium strains show that the lipid portion is the important moiety that allows CD1c-restricted T cells to distinguish between self MPDs and foreign MPMs.[25] Since polyisoprenoid lipids are made by all mammalian cells, T cells have been shown to be specific for particular branched antigens, such as those synthesised by disease-causing *Mycobacteria* species which consist of a *β*-carbohydrate linkage, a C_{30} branched lipid chain containing five methyl groups, phosphates, and mannose groups of *S*-stereochemistry.

Glycosyl Mycolates

Mycolic acids (Chart 1) are *α*-alkyl, *β*-hydroxy fatty acids that are present in most mycobacterium species and also in related taxa-like *Corynebacterium* and *Nocardia*.[26] Mycolic acids from different taxa vary in the number of carbon atoms present: 30-36 for *Corynebacteria*, 40-60 for *Nocardia*, and 80-90 for *Mycobacteria*. The first such acids isolated were obtained by Stodola and colleagues in 1938 from human tubercle bacillus.[27]

Chart 1. Mycolic acids.

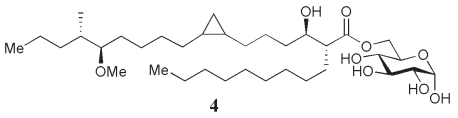


In 1950, Asselineau and Lederer showed that mycolic acids are high molecular weight β-hydroxy fatty acids with a long alkyl chain at the α-position. Subsequent studies revealed that they have at least two stereogenic centres that are located α and β to the carboxylic acid, each with *R* stereochemistry.[28] There are two distinct motifs in mycolic acid: the meromycolate branch and the alpha branch. The alpha branch is similar in every mycobacterial mycolic acid and differs only in chain length. The meromycolate branch, however, is more variable and different acids are classified according to the functionalities found in the meromycolate region.[29] Mycolic acids are restricted to binding to CD1b molecules and were the first lipid antigens found to induce T cell activation via binding to CD1b molecules.[5] Some of these acids have recently been synthesised.[30]

Glucose-monomycolate

Glucose-monomycolate (**4**, GMM), a glycolipid consisting of mycolic acid attached to the 6-position of glucose, is present in numerous bacterial species including *Mycobacterium*, *Rhodococcus* and *Nocardia*. It is a known potent ligand for human T cells when presented by group 1

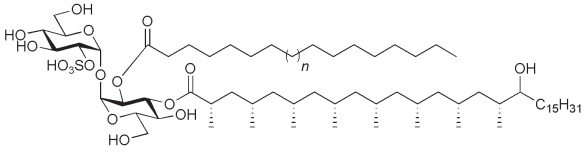
CD1 molecules. Studies have shown that the recognition of GMM by CD1b-restricted T cells is highly specific for the glucose moiety, the *R*,*R* configuration of the mycolic lipid, and the linkage of the mycolate to the glucose unit.[31-33] Variations in the lipid chain of GMM did not change the T cell response to GMM.[31] *Mycobacteria* are unable to synthesise GMM outside the host cell since a non-mycobacterium source of glucose is needed. Accordingly, GMM is only produced by a pathogenic mycobacterium after infection of the host cell.[32] The crystal structure of the CD1b-GMM complex has been solved and shows that both the acyl chains are buried in the antigen binding groove of CD1b, thus leaving the glucose unit exposed to the surface for recognition by TCR.[34] Similar to the CD1a crystal structure, the CD1b-GMM structure shows that a portion of the acyl chain protrudes from the F' pocket.[35]



4

Diacyl trehalose sulfates

The two diacyl trehalose sulfates ($Acyl_2SGL$) **5a** and **5b** have been recently identified as a new mycobacterial antigens that are able to stimulate T cells through CD1 binding.[36] They consist of a trehalose core that is acylated at the 3-position by a hydroxyphthioceranoic acid and at the 2-position by stearic or palmitic acid to give **5a** and **5b**, respectively. In a study by Gilleron *et al*., the addition of $Acyl_2SGL$ to T cells infected with the virulent *MTb* strain H37Rv resulted in intracellular killing of the bacteria via release of IFN-γ. This occurred by stimulation of CD1b-restricted human T lymphocytes by $Acyl_2SGL$, suggesting that $Acyl_2SGL$ or its analogues could be used as subunits in vaccines against tuberculosis.[37] Guiard and co-workers investigated the T cell activation of $Acyl_2SGL$ by varying the length, position, and structure of the fatty-acid residues. The analogues able to stimulate T cells had a saturated or monounsaturated polymethylated fatty acid and the stereocentres at the 3-position of the trehalose were of *S*-configuration. Naturally occurring $Acyl_2SGL$ was most potent in T lymphocyte activation.[38] The length of the fatty acid chain at the 2-position also influences antigenicity. Analogues with C_{16} acyl chains at the 2-position and multi-branched $C_{22\text{-}24}$ lipids at the 3-position stimulated T cells. However, compounds with a short fatty acid chain (C_8) did not lead to T cells stimulation.[39] These studies suggest that $Acyl_2SGL$ can be used as a subunit vaccine against tuberculosis and also in designing lipids that could be used in these vaccines. The first synthesis of diacyl trehalose sulfate and its analogues were reported by Guiard and colleagues[38] in 2008.



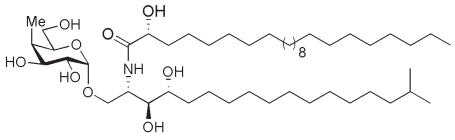
5a, $n = 1$; **5b**, $n = 3$

Glycosphingolipids

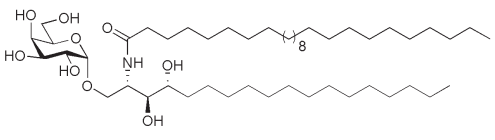
Glycosphingolipids consist of a carbohydrate portion and a ceramide lipid that contains a fatty acid and a sphingosine chain. Aside from α-galactosyl ceramide (α-GalCer), most glycosphingolipids are self antigens able to induce an auto-reactive T cell response. The presentation of self antigens to T cells differs greatly from the presentation of antigens from mycobacterial glycolipids. Glycosphingolipids are presented to T cells without internalization by antigen-presenting cells and they bind to CD1 molecules at physiological pH without the use of chaperone proteins. In multiple sclerosis (MS), an autoimmune response against glycolipid components of myelin, potentially could contribute to disease pathogenesis. It has been shown that T cells of MS patients are more reactive to glycolipids compared to healthy individuals. Self glycolipids, such as sulfatides (*vide infra*), potentially could be the autoantigens that are recognised by T cells in autoimmune diseases.[40]

α-Galactosyl Ceramide (α-GalCer)

In 1993, the pharmaceutical division of Kirin Breweries isolated a series of novel α-galactosyl ceramides from the marine sponge, *Agelas mauritianus*.[41] Of the series, agelasphin-9b (AGL-9b, **6**) was found to be the most potent anti-tumour agent.[42] AGL-9b consists of a galactosyl moiety α1-linked to a ceramide portion containing an *N*-acylated phytosphingosine backbone. Structural optimisation by Morita *et al*.[43] later identified analogue KRN7000 (**7**) as a more suitable candidate for clinical use. The anti-tumour activity of **6** and **7** are comparable, however **7** lacks the hydroxyl group on the acyl chain and the methyl branch on the phytosphingosine backbone. Consequently, its chemical synthesis is more straightforward, thus making it a better drug candidate; KRN7000 is now widely known as α-GalCer.



6, Agelasphin-9b (AGL-9b)

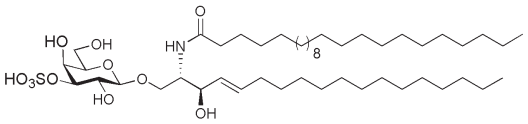


7, KRN7000 or α-galactosyl ceramide (αGalCer)

α-GalCer has been reported to have potential in the treatment of several diseases including cancer, malaria, type 1 diabetes, and multiple sclerosis.[44] The mechanism by which α-GalCer exerts its therapeutic effect only became known with the discovery that it binds to CD1d and activates a subset of T cells called invariant natural killer T (*i*NKT) cells.[45] α-GalCer is the first agonist found to activate the CD1d-restricted *i*NKT cells that express an invariant TCRα chain (Vα24Jα18 in humans and Vα14Jα18 in mice). The first chemical synthesis of α-GalCer was reported by Morita *et al*.[46] in 1995 but subsequently optimised by others.[47,48] Much effort is currently being made to develop derivatives of α-GalCer with improved anti-tumour activity, particularly within the context of cancer immunotherapy, an area of research in which we are particularly interested.

Sulfatide

Sulfatides, illustrated by **8**, are the 3-*O*-sulfate esters of galactosyl cerebrosides and are mainly present in the myelin tissue of mammals (nervous tissue in the central nervous system), although trace amounts are also found in other tissues. The sulfatide glycosphingolipids are essential to myelin and consist of several molecular species that differ in the extent of unsaturation and hydroxylation of the amide-linked fatty acid on the ceramide backbone, and also in the length of the acyl chain.[49]



8, a representative sulfatide

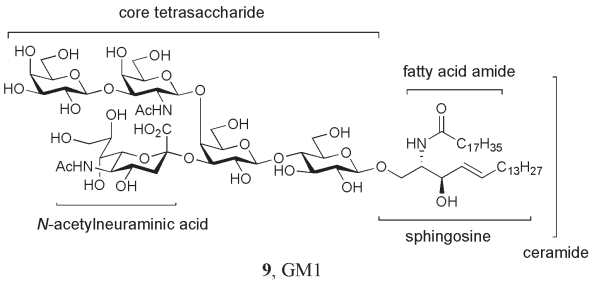
Sulfatides are interesting since they are able to bind to all CD1 isoforms and induce an immune response.[50] CD1a-CD1c all load sulfatides on the cell surface without processing. The sulfatide-CD1a complex persists longer in living cells than the CD1b and CD1c complexes. While the A' pocket in the CD1a binding groove is structured to accommodate alkyl chains (C_{18-23}), the F' pocket (which accommodates the protruding polar head group and most of the fatty acid lipid) is more open and more in contact with the receptor surface of the T cell of the CD1a binding groove, and this likely explains this.[51] CD1a is specially designed to recognize and bind the lipid backbone rather than the head group.

Sulfatide can also bind to CD1d and stimulate natural killer T (NKT) cells. The 3-*O*-sulfate group on the galactose is required for the activation of sulfatide-specific NKT cells, and it has been shown that these are specific for β-linked (but not α-linked) sulfatides.[52] The recognition of sulfatide by the CD1d-restricted NKT cells is important in autoimmune diseases of the central nervous system, particularly multiple sclerosis.[49] The synthesis of sulfatide analogues has been reported by Franchini *et. al*.[52]

Gangliosides

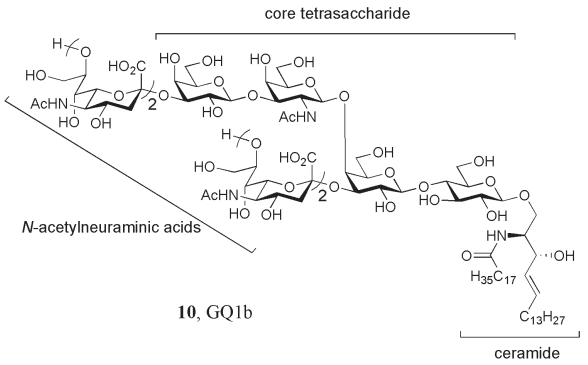
Gangliosides are complex glycosphingolipids found in eukaryotic cells and consist of oligosaccharide chains incorporating *N*-acetylneuraminic acid (NeuNAc) that are attached to a ceramide lipid. They have a β-glucosyl moiety is linked to the primary hydroxyl of the ceramide moiety, and, in turn, a β-galactose is coupled to the glucose 4-position. The structural diversity of this class of compounds results from differences in the sugar moieties that are attached to the β-galactose residue.

GM1:- GM1 (**9**) is the most abundant ganglioside found in human myelin. It consists of the β-galactosyl-β-glucosyl ceramide, to which a β-galactosyl-*N*-acetyl-galactosamine is linked at the 4-position, and an *N*-acetyl-neuraminic acid residue at the 3-position. Unlike most glycolipids that form the glycolipid-CD1 complex intracellularly, GM1 binds to CD1b on the cell surface at neutral pH without prior internalization.[53] GM1 contains the minimum epitope for recognition by T cells for it was found that gangliosides with head groups smaller than GM1 were not stimulatory, while those with larger head groups fully activated T cells. CD1b-GM1 complexes can stimulate T cells to release cytokines such as TNF-α and IFN-γ.[53] Additionally, it has been shown that ganglioside-specific T cells can discriminate between small differences in the carbohydrate portion of the glycolipid and that the terminal galactose of GM1 is important for recognition by T cells.[40] Lyso-GM1 (which lacks the fatty acid amide on the sphingosine backbone) was unable to stimulate T cells, suggesting that both the acyl and sphingosine group of GM1 are required for the binding to CD1b. GM1 binding to CD1b is highly reversible such that other ceramide-containing glycosphingolipids can displace it.



9, GM1

GQ1b:- GQ1b (**10**) is another ganglioside that was found to complex with CD1b and activate T cells. It possesses a core tetrasaccharide to which four *N*-acetylneuraminic acid residues are linked. GQ1b is abundant in the mammalian central nervous system and participates in physiological activities such as toxin binding, modulation of protein phosphorylation, cell adhesion and growth, and apoptosis.[54] In 2001, it was shown that the GQ1b-CD1b complex stimulates T cells and induces the production of cytokines IL-2 and IFN-γ.[55] The first total synthesis of this ganglioside was accomplished in 1994.[56]



10, GQ1b

Conclusion

Glycolipids are important molecules that play a major role in biological processes such as signalling, cell-cell communication, and molecular recognition. In particular, a number of the glycolipids that bind to CD1 have been identified. These include:

- the phosphoglycolipids such as phosphatidylinositolmannoside (PIM), mannosyl-β-1-phosphodolichol (MPD), and mannosyl-β-1-phosphomycoketide (MPM),
- the mycolates, typified by the mycolic acids, glucose-monomycolate (GMM) and diacyl trehalose sulfate ($Acyl_2SGL$), and
- the glycosphingolipids, *e.g.* GM1, GQ1b, sulfatides,

and α-galactosyl ceramide (α-GalCer).

The therapeutic role of glycolipids that modulate the immune system via interactions with CD1 and subsequent T cell activation is still in its infancy. However, much progress is being made in understanding CD1-glycolipid-T cell binding and this has the potential for use of the glycolipids in the treatment of diseases.

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