REVIEW ARTICLE

iNKT-cells and their ligands: focus on multiple sclerosis

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Introduction

The cells of the immune system are broadly divided into those that belong to the innate arm of immunity and those that belong to the adaptive immune system[1] The innate immune cells include macrophages, dendritic cells and neutrophils which express pattern-recognition receptors such as the toll-like receptors, encoded within the germ-line and which are activated by conserved patterns or PAMPs on microorganisms. On the adaptive side of immunity, T and B cells are more diverse expressing receptors encoded by germ-line rearrangement and VDJ recombination which are specifically activated by antigens derived from pathogens. There also exists additional subsets of immune cells which cannot easily be categorised[2] and the division between their involvement in innate and adaptive immunity is less clear cut. These cells include B-1 cells, marginal zone B cells, populations of T-cells bearing the γδT-cell receptor (TCR), mucosal associated invariant T-cells (MAIT-cells) and the invariant natural killer T-cells (iNKT-cells).[2] These populations of T and B cells are more limited in terms of specificity of antigen despite expressing a T or B cell receptor which has been generated by VDJ recombination.[2] This review will focus on the iNKT-cells, on the ligands that activate them and their possible ligand recognition and function in multiple sclerosis.

NKT-cells: general properties and functions

NKT-cells are a unique population of T-cells which were originally identified based upon the co-expression of natural killer (NK) cell receptors such as CD161.[3-6] They represent in mouse about 5% of peripheral blood lymphocytes but are enriched in liver, accounting for greater than 20% of T-cells in some strains.[7,8] There are a

number of subsets of NKT-cells now classified into type I and type II.[9] The majority of NKT-cells in humans express a unique TCR encoded by an invariant Vα24-Jα18 alpha chain gene segments.[5,10,11] Therefore, these are more commonly referred to as invariant NKT-cells (iNKT-cells) or type I NKT while the type II NKT-cells lack an invariant TCR and are more diverse.[9] In addition to CD161, iNKT-cells constitutively express the cell surface markers CD25 and CD69 which are also found on memory and effector T-cells.

Unlike conventional T-cells which respond to peptide antigens in the context of MHC class I or II, iNKT-cells respond and are activated by glycolipid antigens presented by the MHC class Ib related molecule, CD1d.[3,11] CD1d is a highly conserved, non-polymorphic molecule, expressed by many haematopoietic cells (dendritic cells (DCs), macrophages, and B cells) which present lipids antigens rather than peptides to iNKT-cells.[4] The CD1d restriction identifies and defines iNKT-cells from all other T-cell populations.[12,13] Furthermore, development of iNKT-cells in the thymus is dependent upon CD1d and mice lacking CD1d or the Jα18 gene segment lack this population of innate T-cells.[5] After exiting the thymus, iNKT-cells emigrate to the spleen, blood, liver and bone marrow, with smaller populations also found in the intestine.[13] In humans iNKT-cells as identified by CD1d-loaded tetramers account for approximately 0.1-0.2% of peripheral blood T-cells although this percentage is variable.[7,14] The figure is higher in human omentum where iNKT-cells account for up to 10% of T-cells in this location with decreased numbers found in obesity.[15] Alterations in the numbers of iNKT-cells have been correlated with severity of diseases including multiple sclerosis[16,17] and colon cancer.[18] The significance of quantitative changes in the numbers of iNKT-cells may be directly related to the disease pathogenesis or it may simply reflect ongoing immunological activity.

Functions: iNKT-cells are potent and produce an array of cytokines and chemokines in addition to exerting potent cytotoxic activity upon activation. The cytokines produced include IFNγ, IL-2,-3,-4,-10,-13,-17,-21 and TGFβ.[13,19] Stimulation of iNKT-cells by α-galactosylceramide (α-GalCer)(the most potent known ligand found to date) induces production of large amounts of IFNγ and IL-4.[13,20] Moreover the secretion is rapid, with cytokines detectable within minutes to hours of stimulation which differs from conventional T-cells where cytokines are usually

detected after a few days. Subsets of iNKT-cells can also differ in terms of cytokine production: $CD4^-$ NKT-cells produce mainly IFNγ (Th1 cytokine profile) upon stimulation [7,14] while $CD4^+$ NKT-cells produce a mixed Th1/Th2 cytokine profile. Th17-like NKT-cells have also been reported: this population of NKT-cells express IL-23R and produce IL-17A in response to IL-23 mediated activation.[21]

iNKT-cells are activated in two ways: (1) directly, through stimulation via the invariant TCR by glycolipid antigen in the context of CD1d and (2) indirectly, via cytokines produced by antigen presenting cells.[22] In the direct mechanism there are a number of lipid and glycolipid antigens (discussed below) which specifically bind CD1d and activate the semi-invariant TCR on iNKT-cells. iNKT-cells may also however become activated by microorganisms which do not contain lipid and glycolipid moieties as part of their antigenic structure.[19,23] The precise mechanism of indirect iNKT-cell activation still remains to be elucidated but it seems that cytokines such as IL-12 and/or IL-18 produced by the antigen presenting cell and synergised by the presentation of self-antigens by CD1d may be important here.[23]

iNKT-cells play central roles in bridging the innate and adaptive immune responses in microbial infection.[11,24] iNKT-cells have been implicated in immunity to a range of infectious agents, in some cancers, and for suppressive roles in autoimmunity, transplant rejection and graft-versus-host disease.[22, 25,26] iNKT-cells have been shown to play a protective role during infections caused by microorganisms even in the absence of cognate glycolipid antigens. For example during infection with *Streptococcus pneumoniae*, mice lacking iNKT-cells have significantly higher bacterial loads in the affected lungs and a lower survival rate compared with wild type mice.[27] iNKT-cells also contribute to host defence mechanisms against a variety of other infections. In viral infections iNKT-cells are protective and become activated to produce significant quantities of IFNγ particularly when herpes family viruses are involved.[28,29] Moreover, IFNγ producing NK cells and IFNγ in blood were markedly decreased in CD1d-knockout mice which lack iNKT-cells resulting in lower survival rates compared to wild type mice. Nonetheless while there is much evidence to support a protective role for iNKT-cells in microbial infection in some contexts, iNKT-cells may also contribute to the immunopathology associated with some infections. Increased numbers of iNKT-cells have been identified in affected individuals with

chronic lung disease such as chronic obstructive pulmonary disease (COPD) and are believed to have a central role in contributing to the tissue damage here.[30]

Lipid antigens recognised by iNKT-cells

iNKT-cells can bind to a variety of lipid based antigens complexed with CD1d including α-GalCer, exogenous microbial ligands and a growing list of endogenous self-antigens.[5,11,31-33]

α-Galactosylceramide (α-GalCer): The synthetic α-glycolipid, α-GalCer is widely used and is the most well-known lipid antigen used for activating iNKT-cells *in vivo* and *in vitro*.[20] α-GalCer, which is also known as KRN7000 was originally identified for its anti-metastatic properties in a marine sponge sample and became the first known CD1d presented lipid antigen for iNKT-cells.[20,34] α-GalCer has an α-anomeric sugar attached to acyl and sphingosine chains and its ligation to the TCR on iNKT-cells results in internalisation of the TCR and in rapid production of Th1 and Th2 cytokines.[20,34] For many years, α-GalCer was the only antigen which was capable of activating iNKT-cells and its existence in a marine sponge seemed puzzling. Many studies have employed the use of α-GalCer as a therapy based upon its immunomodulatoryproperties.[6] Administration of α-GalCer in mice enhances clearance of a variety of infectious pathogens based upon secretion of pro-inflammatory cytokines by iNKT-cells[25] while in autoimmunity, administration of α-GalCer ameliorates the disease and in this instance is based upon secretion of anti-inflammatory cytokines like IL-10.[6] While previous studies showed that β-glycosylceramides (see below) were the only endogenous anomeric form which induced activation of iNKT-cells, Kain et al.[35] more recently demonstrated that murine immune cells can produce small quantities of α-glycosylceramides.

Exogenous ligands- the microbial glycolipids: In addition to α-GalCer, a number of glycolipids present in the cell walls of bacteria have been identified which stimulate the activation of iNKT-cells (Figure 1). Initial studies demonstrated that

glycosphingolipids(GSLs) from *Sphingomonas spp*. bacteria induced CD1d-dependant activation of iNKT-cells.[26,36-39] In addition to GSLs, further studies demonstrated that glycodiacylglycerols also derived from the *Sphingomonas spp*[37,40,41], and from *Ehrlicha*, and *Borrelia burgdorferi* [42] (which causes Lyme disease) stimulated iNKT-cells although they varied widely in their antigenic potency. The list of microbial lipid antigens also includes diacylglycerol-containing glycolipids from *Streptococcus pneumoniae*, and from Group B *Streptococci* [42].These are clinically important pathogens capable of causing invasive diseases such as pneumonia and neonatal sepsis and meningitis.[26,43] A tetra-mannosylated form of phosphatidylinositol called PIM4 from *Mycobacterium bovis*[44] is another foreign lipid antigen capable of activating iNKT-cells. More recently, Change et al.[28] identified that a cholesteryl-α-glucoside from *Helicobacter pylori* can stimulate iNKT-cells. Therefore it appears that iNKT-cells exhibit a broad range of ligand specificity. The main difference between the microbial lipid antigens identified so far which activated iNKT-cells and the mammalian lipid antigens is that microbes produce α-linked glycolipids whereas mammals generate only β-linked lipid antigens.

There is also other exciting data which suggests that iNKT-cells sense infection and respond preferentially to inflammatory stimuli such as cytokines (e.g IL-12) despite the presence of known microbial antigens.[29,45] Interestingly the response requires the conversion of isoglobotrihexosylceramide 4(iGB4) to the self -agonist iGB3 (see below) by β-hexosaminidase.[46] Overall in microbial infection iNKT-cells have many mechanisms therefore by which to respond and become activated bysensing microbial infection indirectly through inflammatory cytokine production and possibly through the recognition of CD1d restricted self-antigens.

Synthetic lipid antigens: The discovery that α-GalCer had potent iNKT-cell activating properties associated with rapid Th1/Th2 cytokine secretion led to several subsequent studies investigating the stimulatory effects of alternative synthetic analogues (reviewed in Venkataswamy & Porcelli [47]; Tyznik et al.[48]). The aims of these investigations were to identify synthetic analogues which were capable of inducing either a Th1 or a Th2 cytokine response and which could be administered

therapeutically when treating conditions such as cancer, allergy and autoimmunity where polarised cytokine responses were implicated in pathogenesis.

These synthetic ligands identified include the sphingosine-based truncated derivative of α-GalCer such as OCH, the N-acyl-derivatives such as C:20, the glycosidic bond derivatives and the carbohydrate-modification-based derivatives.[47] OCH induces a Th2 response during activation of iNKT-cells in mice as defined by rapid IL-4 production with no detectable IFNγ.[49] Other sphingosine-based compounds have been synthesised where the amide of the ceramide base of α-GalCer was replaced by a triazole 8 group which *in vitro* and *in vivo* was as potent an activator as α-GalCer.[47,50] Other analogues of α-GalCer have been made by altering the length and the degree of unsaturation of the N-acyl substitution including C20:2 analogue which is also a very potent inducer of Th2 cytokines. [47,51]The list of synthetic lipid antigens for iNKT-cells is growing and their capacity to induce biased immune responses holds great promise therapeutically. [52]

The endogenous self-antigens: ligands for iNKT-cells

One of the defining functional characteristics of NKT-cells is autoreactivity or their ability to react to self-antigens; a property identified by response to CD1d-expressing antigen-presenting-cells in the absence of exogenous antigen.[53] While there is still much to be learned and much controversy in this area, a variety of self-lipid antigens have now been identified as being stimulatory for iNKT-cells. The initial search for an endogenous self-antigen using mouse iNKT-cell hybridomas identified β-glucocosylceramide as being a murine iNKT-cell agonist[54] though far less potent than α-GalCer. While these self-antigens are mainly glycosphingolipids the list also includes a variety of phospholipids. Furthermore as discussed above, the capacity of iNKT-cells to sense and respond to bacteria indirectly is also most likely because of their recognition of self-antigens during infection.[45]

The self-glycosphingolipids iGB3 is an isoglobotrihexosylceramide, a lysosomal β-linked glycosphingolipid identified by Zhou et al.[32] as the first endogenous glycolipid activator of human NKT-cells. iGB3 has been shown to activate mouse

iNKT-cells via CD1d and mice deficient in iGB3 have defective iNKT-cell development.[32] The crystal structure of iGB3 bound to CD1d has been elucidated and describes the mechanism of how this self-lipid is recognised and is stimulatory for NKT-cells.[55] Although it is reported as a potent activator of both human and mouse iNKT-cells, its significance as a self-antigen for iNKT-cells is contentious and remains under debate.[56] In murine studies several deficiencies of enzymes involved in lipid metabolism were shown to have profound impacts on iNKT-cell development[57]and this includes deficiencies of hexosamidase B which is the enzyme involved in synthesis of iGB3. Furthermore in human studies, the functional iGB3 antigen is not present on mammalian cells[58] because the relevant iGB3 synthase which generates it is absent.[59] Moreover in humans iGB4 can be converted to iGB3[46] although its identity as a dominant self-ligand for iNKT-cells is still unclear. In addition to iGB3, the ganglioside GD3 which is highly expressed on tumours of neuroectodermal cells, is stimulatory for iNKT-cells[60] and there are additional glycosphingolipids (GSLs) identified within the central nervous system which also are stimulatory for iNKT-cells and these are discussed below in the context of autoimmunity of the central nervous system.

The self-phospholipids: In addition to the GSLs, other studies have identified phospholipids to be stimulatory self-antigens for NKT-cells. These include phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine[3,47] which were shown to activate a small percentage of iNKT-cells from hybridomas in a CD1d-dependant manner.[3] While phospholipids are proposed to be self-antigens for iNKT-cells, their stimulatory activity and their potency is weak compared with the α-linked lipid antigens, and has only been demonstrated for a subset of iNKT-cells

In studies investigating lipids eluted from CD1d, Fox et al.[61] identified lysophosphatidylcholine as a self-antigen based upon its reactivity for a subset of human iNKT-cells. In an earlier study[62] lysophosphatidylcholine was isolated from the plasma of patients with multiple myeloma and was also shown to stimulate activation of iNKT-cells. Lysophosphatidylcholine contains a single fatty acid tail and is known to accumulate in human inflammatory conditions such as human autoimmunity, asthma and cancers where phospholipases are activated. In contrast to the cytokines secreted by NKT-cells such as IFNγ and IL-4 in response to other stimuli,

iNKT-cells stimulated by lysophospholipids secreted only GM-CSF.[61] More recently, lysophospholipids were shown to activate type II NKT-cells which lack the invariant TCR as potently as sulphatide and their activity me be important in inflammatory liver disease.[63] Plasmalogen lysophosphatidylethamolamine (plasmalogen lysoPE) is a glycerol-based lipid with a single fatty acid chain which was identified as a self-antigen for iNKT-cells in studies investigating the nature of lipid antigen involved in their thymic selection.[64]

Lipid antigens and iNKT-cells in Multiple Sclerosis (MS)

iNKT-cells through their rapid secretion of cytokines can exhibit both pro- and anti-inflammatory properties and this decision is dependent upon how they become activated, and the cells and cytokines in the local environment.[2,19] iNKT-cells have been shown to have either protective or harmful roles in many pathological states and it is difficult to predict how these innate lymphocytes will function during an immune response. In many autoimmune disorders, defects in the numbers and/or the functions of iNKT-cells have been identified.[65] In some animal models of autoimmunity while NKT-cell-deficiency exacerbates disease, suggesting that iNKT-cells play a role in suppressing autoimmunity, specific activation of iNKT-cells with glycolipid antigens generally protects mice against the development of autoimmunity.[6] In autoimmunity, iNKT-cells exert their immuno-regulatory functions through release of cytokines, activation of immune cells and induction of cytolytic activities.[66]

Multiple Sclerosis (MS): MS is the most common demyelinating disease in man and while the main target autoantigen is myelin, other central nervous system cells including neurons, their axons, microglia, endothelial cells and pericytes may also be affected.[67,68] The demyelination in MS is pro-inflammatory with the pathological and tissue damaging events being due to activation of Th1 cells and the secretion of inflammatory cytokines like IFNγ and TNFα.[69] Several immune cell abnormalities have been described in MS[70] including changes in the numbers of iNKT-cells[16,17,71,72] although the relative contributions of individual regulatory cell subsets remains to be clearly defined.

The myelin sheath is a unique, multi-layered membrane which is rich in lipids and glycolipids in particular complex glycolipids, such as galactosylceramides, gangliosides, sulphatides and phospholipids.[73] Previously, we characterized a novel mammalian brain GSL series that accounts for 15–35% of total myelin GSL content, and which is designated ‘fast-migrating cerebrosides’(FMC) on the basis of TLC migration[74-76] (See Figure 2). These acetyl-monogalactosyl-GSL include simple and more complex compounds ranging from penta-to hexa-acetylated derivatives ofβ-galactosylceramide (i.e. the‘cerebrosides’). Stimulatory studies indicated that the myelin derived polyacetylated β-galactosyl-ceramides (PA-GC) were potent iNKT-cell activators in peripheral blood of healthy humans and this was in marked contrast to the findings in MS.[72] The PA-GC (a mixture of FMC-5 and FMC-7)[74-76] and in particular, the purified FMC-7 induced proliferation and cytokine secretion as potently as α-GalCer.[72] FMC-7 is an endogenous mammalian and CNS derived acetyl-glycolipid that contrasts in its structure from α-GalCer by having a β-linked galactose rather than an α-linked galactose bound to ceramide. From our molecular modelling the acetylation modifies the conformation of the galactosylceramide that is characterized by free rotation of the galactose about the C-1 of ceramide by hydrogen bridge formation between the acetylated 3-OH-sphingosine and theacetylated 2-OH-galactosyl and this may constrain the C-1 rotation.[73] We propose that this alters the conformation of the polyacetylated FMC-7 acetyl-galactose head-group to fit the iTCR and then initiates the activation of the iNKT-cell: a speculation consistent with current concepts of ‘glycolipid moulding’ in the CD1-glycolipid-iTCR synapse. Thus myelin-derived PA-GC are potentially self- reactive endogenous ligands for iNKT-cells amongst freshly isolated peripheral blood lymphocytes from healthy control subjects.

Anergy amongst iNKT-cell populations has previously been demonstrated in mice employing repeated injections of α-GalCer that induces unresponsiveness to re-stimulation *in vivo* or *in vitro*.[77] Hyporesponsivness or anergy of lymphocytes from MS patients in remission has previously been reported[78] with poor proliferative capacity that was broken with CD28 stimulation while regulatory T-cells had unaltered suppressive function except for lower IL-7 receptors (CD127). In our studies, using PA-GC and FMC-7 as stimulatory ligands, iNKT-cells from MS patients failed to respond *in vitro* to stimulation which was in marked contrast to the broad range of

Th1, Th2 and Th17 cell cytokines produced by healthy controls.[72] This hyporesponsivess of the iNKT-cell population was also observed in response to α-GalCer stimulation[16] and importantly in our study this anergic glycolipid response as measured by an expansion in cell number was specific to the iNKT-cell population.[72] The identification of myelin-derived lipid antigens that anergise iNKT-cells which may participate as effector cells is central towards developing an understanding the complexity of this neurodegenerative disease. We note however that these lipid antigens are not present at many sites where iNKT-cells accumulate and therefore it is difficult to appreciate fully their role in immunity. In MS, moreover, the concentrations of these myelin-derived PA-GC are altered[74] raising the possibility that these myelin GSLs contribute to an iNKT-cell role *in vivo*. The mechanisms responsible for anergy development in response to stimulation with PA-GC remain to be determined although altered signalling mechanisms through the TCR and/or PD-L1 [79.80] may be important. Other studies have demonstrated that tolerance to α-GalCer does not depend upon IL-10, caspase-3-mediated apoptosis or T regulatory cells.[81]

It may be that in MS continuing destruction of the myelin sheath increases the exposure of myelin-derived lipids in context of CD1d thus inducing inactivation or anergy of iNKT-cells through mechanisms not yet clarified. These myelin GSLs may contribute to iNKT-cell autoreactivity in MS in a fashion similar to the way another lipid self-antigen, β-glucosylceramide acts during microbial infection.[45] Moreover, the synergistic action of cytokines and the TCR stimulation of iNKT-cells by self-antigens such as PA-GC and/or foreign lipid antigens may also lead to the anergic phenotype of iNKT-cells in MS. This synergistic recognition of self and foreign lipid antigens by iNKT-cells has been demonstrated to shape the iNKT-cell compartment during microbial infection.[22] The relative contribution of several factors including subsets of iNKT-cells, cytokines, the PA-GC concentrations as well as possible microbial infection and ongoing autoimmune phenomena is likely to be context dependant. Nonetheless, unravelling the complexities of iNKT-cell function in MS and the possibility of rendering iNKT-cells hyporesponsive to an endogenous glycolipid is a novel mechanism for iNKT-cell regulation in disease. This glycolipid ligand-driven anergy has substantial implications for MS immunotherapy.

Conclusions

Recent progress in studies of glycolipid and glycolipid-induced findings in identifying the nature of lipid recognition for iNKT-cells in immunity and in determining the functional consequences of the lipid-CD1d interaction for this important iNKT-cell population opens new avenues of access to the pathogenesis of demyelination in MS. The list of relevant lipids and glycolipids including self-antigenic ligands for iNKT-cells continues to grow and includes iGb3[33] and in microbial infection β-glucosyl-ceramide[45] and in human MS, the myelin-derived PA-GC.[72] These advances lead to a number of outstanding questions pertinent to the functional roles of iNKT-cells in autoimmune diseases such as MS, and include the following: what mechanisms determine whether iNKT-cells will remain self-tolerant versus becoming self-reactive and potentially tissue damaging? are there functional differences in subpopulations of iNKT-cells in terms of their self-ligand antigenic specificities which may be central to autoimmunity? what is the molecular basis for the anergic response of iNKT-cells during an autoimmune response? A deeper understanding of the endogenous self-antigens targeted by iNKT-cells is likely in the future to foster the development of therapeutic strategies aimed at harnessing iNKT-cell activity.

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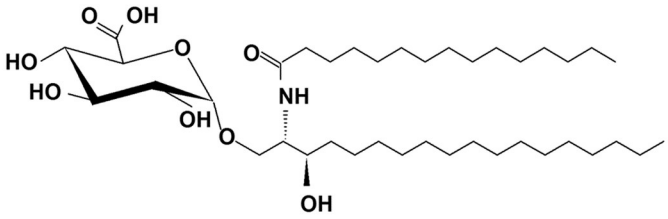
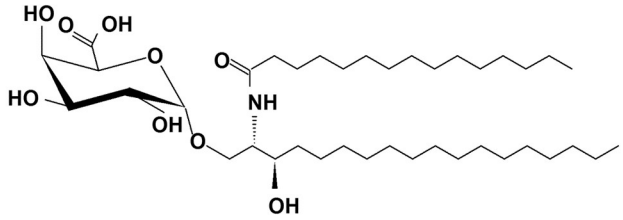
A



α-GlcACer

B



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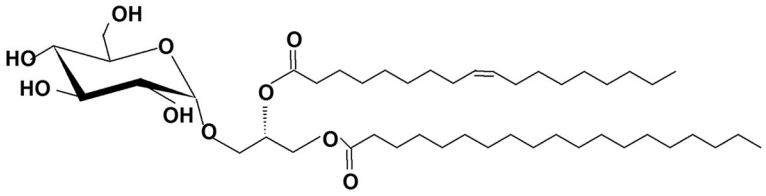

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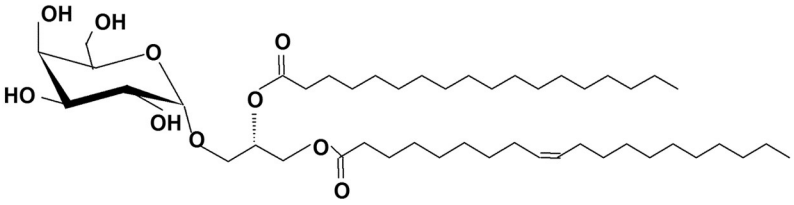

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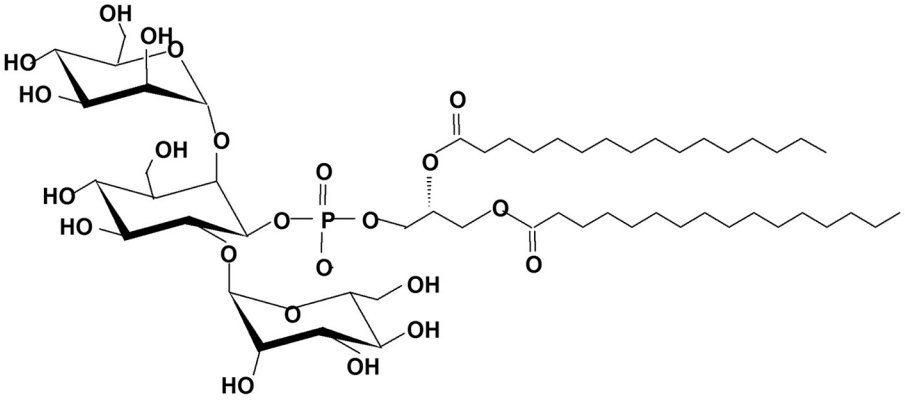
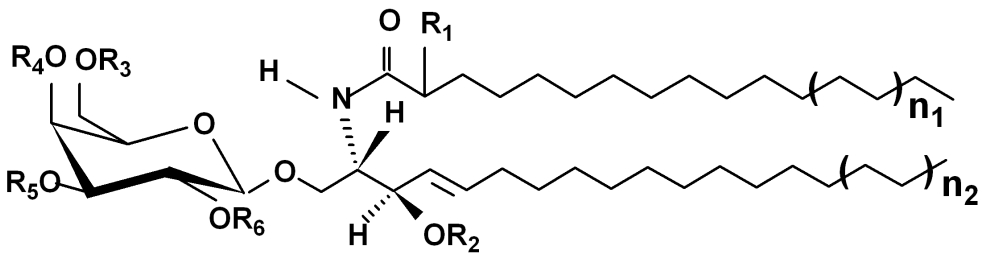


Figure 1

Chemical structures of various microbial lipids that bind CD1d. These include α-galactosyldiacylglycerol (αGalDag) from *Borrelia burgdorferi*, α-glucosyldiacylglycerol (α-GlcDAG) from *Streptococcus pneumoniae*, α-glucuronsylceramide (α-GLcACer), α-galacturonosylceramide (α-GalACer), and phosphatidyl-myo-inositol mannoside (PIM2).



β-GalCer: R1=H, R2=H, R3-R6=H;
FMC-5: R1=H, R2-R6=Ac;
FMC-7: R1=OAc, R2-R6=Ac.

Figure 2

Structure of the myelin-derived fast migrating cerebrosides (FMC). The basic glycosphingolipid structure shows where the R groups are positioned and includes a list of the substitutions giving rise to β-GalCer, FMC-5 and FMC-7 (Adapted from Podbielska et al, 2010).

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