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NKT Cell Ligand Recognition Logic: Molecular Basis for a Synaptic Duet and Transmission of Inflammatory Effectors[1]

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Abstract

Natural killer T (NKT) cells that express the semiinvariant T cell receptor are innate-like lymphocytes whose functions are regulated by self and foreign glycolipid ligands presented by the antigen-presenting, MHC class I-like molecule CD1d. Activation of NKT cells in vivo results in rapid release of copious amounts of effector cytokines and chemokines with which they regulate innate and adaptive immune responses to pathogens, certain types of cancers and self-antigens. The nature of CD1d-restricted ligands, the manner in which they are recognised and the unique effector functions of NKT cells suggest an immunoregulatory role for this T cell subset. Their ability to respond fast and our ability to steer NKT cell cytokine response to altered lipid ligands make them an important target for vaccine design and immunotherapies against autoimmune diseases. This review summarises our current understanding of CD1d-restricted ligand recognition by NKT cells and how these innate-like lymphocytes regulate inflammation.

Introduction

The immune system evolved with the descent of multicellular metazoans as a means to recognise and respond to altered internal milieu (homeostasis). Both internal and external stressors–such as toxic substances or microbial and parasitic infections–are known to incite tissue injury. Containment and removal of the stressor–which are essential for initiating tissue repair–are accomplished initially by the archaic, multi-modular innate immune system. The innate-like lymphocyte module consists of natural killer (NK), B1 B, γδ T, natural killer T (NKT) cells, and others. NKT cells have evolved to jump-start and fine-tune the innate and adaptive immune responses. The adaptive immune system consists of B and T–lymphocytes which are recruited to assist in the healing process should the innate mechanisms fail to contain and clear the inciter. The quick-acting, innate system senses an altered homeostatic state with pattern recognition receptors. In contrast, the slow-responding, adaptive immune system uses antigen-specific receptors that are expressed clonally by B and T lymphocytes–B cell receptors (and Abs), and TCRs, respectively–to sense alterations in the internal milieu. Whilst each module plays a specific role, multiple modules act in concert resulting in an inflammatory response that is essential in maintaining homeostasis (reviewed in ref. 1). In this review, we discuss the current knowledge of a duet

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between NKT cells and APCs–pivotal to which is the understanding of the TCR-ligand recognition logic–and its impact on inflammation.

NKT cells

NKT cells–which express both NK and T cell phenotypic and functional features–are thymus-derived, innate-like lymphocytes whose functions are regulated by self and non-self lipid ligands presented by CD1d molecules. CD1d molecules are expressed by APCs–such as dendritic cells (DCs), macrophages (Mφ) and B cells–as well as $CD4^+8^+$ thymocytes, hepatocytes and intestinal epithelial cells. Hence, under different experimental and pathologic conditions, each of these $CD1d^+$ cell types can present self and microbial lipids and activate NKT cells (2–12).

The majority of NKT cells express an invariant TCR α-chain generated by TRAV11*02 (mouse Vα14i) or TRAV10 (human Vα24i) to TRAJ18 (Jα18) rearrangement. The invariant α-chain pairs predominantly with mouse TRBV13–2*01 (Vβ8.2), TRBV29*02 (Vβ7), TRBV1 (Vβ2) or human TRBV25–1 (Vβ11) β-chain to form a functional semiinvariant TCR. A small subset–referred to as type II NKT cells–expresses a more diverse TCR repertoire but little is known regarding their properties, and hence not discussed here. NKT cells regulate microbial and tumour immunity as well as autoimmune diseases by their ability to rapidly secrete large amounts of immunoregulatory cytokines and to upregulate costimulatory molecules to alert and modulate the effector functions of myeloid and lymphoid cells (13, 14).

CD1d-restricted ligands and distinct modes of NKT cell activation

Exogenous NKT cell agonists—CD1d is a member of the CD1 family of antigen presenting molecules. The original report by Brenner and colleagues demonstrating CD1-restiction of *Mycobacterium tuberculosis*-reactive T cells and the recognition of *Mtb* lipids indicated that CD1 molecules present lipid ligands (15–18). Consistent with this is the finding that CD1d assembles with cellular phospholipids and sphingolipids (19–23). CD1d then acquires self and microbial NKT cell agonists in the endo/lysosomes (24). Much of our understanding of NKT cell biology however, has been gleaned from numerous in vitro and in vivo studies that use the marine sponge-derived, synthetic (KRN7000) αGalCer and its analogues as the probe (Fig. 2, 3; (13, 14, 24–29). *Sphingomonas* spp.–a Gram-negative α-Proteobacteria that lack LPS–synthesise α-glucuronosylceramide and α-galacturonosylceramide (αGalACer) that resemble αGalCer (Fig. 2). αGalACer directly activates NKT cells in a CD1d-restricted manner (30–32). NKT cells activated byαGalACer appear to be important in *Sphingomonas*-specific immunity because high-dose infection of wild-type mice results in septic shock caused by rapid release of inflammatory cytokines, whilst low-dose infection of NKT cell-deficient mice delays bacterial clearance (30, 31).

NKT cells also recognise diacylglycerol-based microbial lipids; e.g.,α-galactosyldiacylglycerol (αGalDAG) and phosphatidylinositoltetramannoside ($PtdInoMan_4$) (Fig. 2) (33, 34). These glycolipids are cell-wall components or theirprecursor synthesised by *Borrelia burgdorferi* (35, 36)–the agent of Lyme disease–and *Mtb* (34), respectively. Additionally, NKT cells are activated by *Helicobacter pylori*-derived cholesteryl-6-O-acyl α-glucoside (37). Hence, NKT cells have broad ligand specificity. Whilst how structurally distinct ceramide- and diacylglycerol-based glycolipids are recognised by the semiinvariant NKT cell receptor (NKTCR) is discussed below, how the cholesterylglucoside is recognised remains unknown.

Endogenous NKT cell agonists—NKT cells are also autoreactive–i.e., they react to self lipids presented by the host APCs (38, 39). An initial search for the endogenous NKT cell

agonist revealed that neither cells deficient in β-glucosylceramide (βGlcCer) synthase and transiently expressing CD1d nor cell-free CD1d-βGlcCer complexes activate mouse NKT cell-derived hybridomas (40). This finding suggested that one endogenous mouse NKT cell agonist is a cellular, βGlcCer-derived glycosphingolipid (GSL). Notwithstanding, current evidence suggests that both β-linked GSLs–e.g., cellular βGlcCer, isoglobotrihexosylceramide (iGb3; Fig. 2), GD3 and an analogue, β-mannosylceramide (41–44)–as well as glycerophospholipids–e.g., Ptd-inositol, Ptd-ethanolamine and lyso-Ptd-choline (45–47)–are agonists for a subset or all mouse and/or human NKT cells. The identity of other self ligands await identification and characterisation.

The importance of self lipid recognition was realised in studies demonstrating human and mouse NKT cell activation by DCs co-cultivated with either the Gram-positive *Staphlococcus aureus* or the Gram-negative *Salmonella typhimurium*. In the case of *S. typhimurium*, NKT cell activation resulted from bacterial LPS-mediated stimulation of DCs through TLR4 and the secretion of IL-12 (48). This response requires hexosaminidase B (HexB), which converts, amongst other GSLs, the precursor iGb4 to agonistic iGb3 (Fig. 1). These data were interpreted to mean that *Salmonella* activates NKT cells indirectly through the recognition of self-iGb3 in the presence of IL-12 (31).

Whether iGb3 is the sole endogenous NKT cell agonist has been contentious as NKT cells from iGb3 synthase-deficient mice are fully functional (49). Additionally, only mouse dorsal root ganglion but neither human thymocytes nor DCs appear to synthesise iGb3 (49). Nonetheless, human thymocytes synthesise iGb4 (50) and HexB (44), which can convert iGb4 to iGb3. Moreover, iGb3 is detectable in the absence of the regioisomer Gb3 (51) suggesting that iGb3 is ephemeral and that its levels are regulated by either rapid anabolism to iGb4 or catabolism by the lysosomal α-galactosidase A (GalA; Fig. 1). Indeed, APCs deficient in GalA–which cleaves reducing α-linked Gal residues–cause overt activation of wild type NKT cells, suggesting the accumulation of an agonist (52). GalA also converts iGb3 (and Gb3) to lactosylceramide and therefore, its deficiency increases iGb3 levels in cells up to five-fold (51). A caveat with experiments that use cells or cell lines deficient in lipid metabolic enzymes is that, they are known to cause lipid storage disease, which can in turn alter lipid and protein trafficking within cells (53–57). Nonetheless, these findings have implications for the role of NKT cell responses to microbial infections as *Salmonella*-infected cells or those stimulated by microbial products that down regulate *GalA* expression (52) thereby, increasing the levels of iGb3.

NKT cells also respond to a sialylated endogenous lipid when DCs are activated by CpG, a TLR7 ligand, and produce IFN-α (58). They also respond to a combination of inflammatory cytokines such as IL-12 and IL-18 in the absence of a CD1d-restricted agonist (59–61). This latter mechanism is important for immunity to cytomegalovirus (61). Hence, NKT cells have evolved multiple ways to sense microbial stressors including direct recognition of CD1d-restricted exogenous glycolipids. Alternatively, they sense stressors indirectly, either through the recognition of CD1d-self lipid complex or in a CD1d-independent manner, in the presence of inflammatory cytokines.

Structures of CD1d lipid complexes

CD1d is a heterodimer consisting of a heavy chain that is noncovalently associated with the light-chain β2-microglobulin. The heavy chain folds into five domains: the extracellular α1, α2 and α3 domains (Fig. 4A), which are membrane-anchored by the transmembrane region, ending in a short cytoplasmic tail. Solution of the three-dimensional structures of mouse and human CD1d molecules, which differ subtly from each other, in complex with several lipid ligands–αGalCer, αGalACer, OCH, αGalDAG, sulfatide, $PtdInoMan_2$, PtdCho, iGb3 (8, 62–69)–revealed that the α1 and α2 domains of the heavy chain fold into a superdomain to form

the Ag-binding groove (ABG; Fig. 2–4). The ABG is laterally confined by two antiparallel α-helices that are supported at the bottom by an 8-stranded antiparallel β-sheet platform. The membrane-proximal immunoglobulin-like α3 domain and the noncovalently associated light chain support the superdomain (Fig. 4A). Therefore, the topology of CD1d resembles peptide-antigen-presenting MHC class I molecules.

Display of αGalCer and its analogues—The arrangement of the amino acids that make up the ABG is such that the narrow apical entrance leads into two deep-seated pockets (A′ and F′; Fig. 2, 3). The two pockets are lined predominantly by hydrophobic amino acid residues, and hence permit the binding of hydrocarbon tails of lipid molecules of varying lengths. The *N*-acyl chain of αGalCer and related compounds–OCH and αGalACer–tucks into the large A′ pocket while the long-chain base of GSLs fits into the F′ pocket (Fig. 2, 3). This binding mode exposes the polar head group out from the ABG (Fig. 3, 4). Moreover, the charged amino acids at the entrance of the ABG form a conserved hydrogen-bond network with polar atoms of the head groups of these α-anomeric GSLs (8, 62, 63, 66–68). The same CD1d residues also form hydrogen-bonds with β-anomeric GSLs, such as sulfatide–a type II NKT cell ligand–and iGb3 (68, 69). This hydrogen-bond network provides stability to the CD1d lipid interaction. Thus, the physicochemical architecture of the ABG dictates how the polar epitope is disposed for recognition by the Vα14i/Vα24i TCR, or the TCR of more diverse type II NKT cells, in the case of sulfatide.

Display of αGalDAG—Microbial αGalDAG antigens are structurally similar to αGalCer in that they also have an α-anomeric galactose attached to a lipid backbone (Fig. 2). However, in contrast to αGalCer, the DAG backbone is characterized by two fatty acids esterified to both the *sn*-1 and *sn*-2 position of a glycerol moiety, while the α-anomeric galactose is attached to the *sn*-3 position. Borrelial αGalDAG lipids bind to CD1d in two different orientations, depending on the nature of the acyl chains linked to *sn1*- and *sn2* positions of glycerol. As a result, the lipid backbone is important in the formation of a TCR epitope as certain αGalDAGs are NKT cell agonists and others not, because they bind in the opposite orientation (65). *B. burgdorferi* glycolipid 2c (BbGl-2c), which is bound with the *sn*-1 oleic acid ($C_{18:1}$) in the A′ pocket and the *sn*-2 palmitic acid ($C_{16:0}$) in the F′ pocket is a mouse NKT cell agonist (Fig. 4D), whilst BbGl-2f that binds in a reversed orientation with the *sn*-2 linked oleic acid ($C_{18:1}$) in the A′ pocket and the *sn*-1 linked linoleic acid ($C_{18:2}$) in the F′ pocket does not activate mouse NKT cells (33). In contrast, BbGl-2f is a human NKT cell agonist (33), but how αGalDAGs are presented by human CD1d is currently unknown. Even though chemical modifications, such as unsaturations do not directly make contact the TCR, but by virtue of affecting the orientation of the hexose sugar contributes to the formation of the NKT cell epitope. Similar changes in the ceramide backbone of αGalCer analogues do not lead to alternative GSL binding orientation, as the ceramide backbone is bound in a conserved orientation through the conserved hydrogen bond network. In sum, αGalDAG presentation reveals for the first time striking differences between mouse and human glycolipid antigen recognition that could not have been appreciated by using strong agonists, such as αGalCer.

Self display: sulfatide and iGb3—Sulfatide binds CD1d in a distinct manner such that the 3′-sulphated galactose is solventexposed and projectsup and away from the ABG as a result of its β linkage (Fig 1, 2; (68). This contrasts the more intimate binding of the galactosyl headgroup of αGalCer to CD1d (8, 63). However, despite differences in the binding mode, sulfatide engages CD1d via a hydrogen-bond network mediated by the same residues involved in stabilizing αGalCer (8, 63, 68).

The first hexose of iGb3–i.e., glucose–akin to sulfatide, is β-linked to ceramide, and hence would be predicted to be solvent exposed in a manner similar to the sulphated galactose.

This disposition of the first glucose of iGb3 results in an almost perpendicular exposition of the two terminal galactoses [Glc β(1–4)Gal α(1–3)Gal] of iGb3 out of the ABG as revealed by the structure of the mouse CD1d iGb3 complex (Fig. 2, 3) (69). Nevertheless, the β-linked glucose, which unalike sulfatide lacks a 3′-sulphate and whose 4′-hydroxyl is equatorially disposed, perhaps results in poor binding to CD1d because the 3′-sulfate and the axial 4′-hydroxyl are involved in hydrogen bonding of sulfatide with CD1d.

Taken together, the presentation principles for α- and β-linked glycolipids are distinct. How then the same Vα14i/Vα24i TCRs recognise these structurally distinct agonists remains to be elucidated. Finally, it will be interesting to see why sulfatide and iGb3, which share the same core-structure, are recognised by different NKT cells: sulfatide being a type II NKT cell agonist whilst, the latter a semiinvariant NKT cell agonist.

Display of phosphoglycerolipids—Lyso-Ptd-choline, but not Ptd-choline, is a human NKT cell agonist (45). As it consists of only one acyl-*sn1*-glycerol, it will be interesting to see into which pocket this single chain lipid binds or, whether two different binding orientation exist of which only one orientation results in an agonist, similar to what has been observed in the case of the borellial DAG ligands (65). As most agonists that are structurally characterized in complex with the NKTCR contain an α-linked galactose that show a conserved TCR binding footprint, it is difficult to predict how the more complex glycolipids, such as PIM_4 or iGb3 are recognized and engaged by the same NKTCRs.

NKTCR/CD1d-lipid recognition logic

By contrast to TCR/pMHC complexes–wherein the receptor docks diagonal on the antigen (70)–the NKTCR docks parallel onto the extreme C-terminal end of the CD1d ABG, above the F′ pocket by using three of the six CDRs–CDR1α, CDR3α and CDR2β–while almost excluding CDR2α, CDR1βand CDR3β from the interface (Fig. 4; (71–73). This docking mode enables a lock-and-key interaction with the α-linked galactose epitope that was predicted from biophysical studies of Vα14i TCR ligand binding (71–74). Furthermore, alanine-scanning mutagenesis of the mouse Vα14i TCR as well as the crystal structures of Vα14i-Vβ8.2 and Vα14i-Vβ7 co-complexed with mouse CD1d-αGalCer revealed that the mouse NKTCR interfaces its ligand in a manner similar to the Vα24i TCR ligand interaction (72, 73, 75–77).

The above germline-encoded recognition logic raises the question of how the mouse Vα14i and human Vα24i TCRs recognise structurally distinct ligands such as iGb3, GD3, $PtdInoMan_4$, PtdIno, PtdEtN and lysoPtdCho. Alanine-scan mutants of Vα14i TCR revealed that the NKTCR recognises many α-linked GSLs (αGalCer, OCH, αGalACer, αGalDAG and iGb3, which contains an α-linked terminal galactose) by means of a ‘hot-spot’ of germline-encoded amino acids within CDR1α, CDR3α and CDR2β loops (77). The recent structure of mCD1d-PtdIno bound to an autoreactive Vα14i TCR, in which the β-chain has been mutated to increase affinity toward self-antigens, surprisingly revealed that CDR3α residues do not directly contact the glycolipid, although the conserved TCR footprint on CD1d is maintained, while additional residues in CDR2α contact the phosphoinositol headgroup (Fig. 4; (78). Those interactions are novel and have not been reported for any other Vα14i TCR. Whether recognition of glycolipids by CDR2α residues is unique to self-antigens, however, is currently unknown. Additionally, the recent solution of the Vα14i-Vβ8.2/mouse CD1d-αGalDAG and αGalACer crystal structures revealed that the TCR has the capacity to induce structural changes in both CD1d and the ligand orientation to maintain the conserved TCR footprint (Fig 4B-E; (72). Similar to αGalCer and αGalACer, the TCR contacts αGalDAG exclusively through CDR1α and CDR3α (72). In each of these ternary structures, CDR1α Asn30 hydrogen bonds with the 2′ and 3′ hydroxyls of the

galactose or galacturonic acid of the GSL (72). However, for αGalDAG this conserved interaction with the TCR required a re-orientation of the galactose moiety (Fig. 4E; (72). CDR3α residue Gly96 contacts the 2′-OH through a main chain carbonyl, whilst Arg95 contacts the 3″-OH of the ceramide backbone of both αGalCer and αGalACer (72). However, this hydrogen bond interaction is lost in the αGalDAG structure due to the different lipid backbone structure (72). These findings suggest that the interaction of NKTCR with structurally distinct α-linked ligands is accomplished by similar recognition logic, which involves the germline-encoded 'hot-spot' composed of amino acids within CDR1α, CDR3α and CDR2β loops.

The recently determined crystal structures of nine ternary complexes with bound αGalCer analogs, such as OCH, C20:2 αGalCer, C20:2 αGluCer, 3′,4″-deoxy αGalCer, 4′,4″-deoxy αGalCer (79), as well as C-glycoside, BnNH-GSL-1′, and naphtylurea (NU)αGalCer (80), provide insights into the mechanisms of TCR binding, as well as illustrates novel and unexpected findings about the flexibility of the antigen presenting molecule CD1d. The successive elimination of individual hydroxyl groups of αGalCer analogues at either the galactose moiety or the phytosphingosine chain disrupts individual H bond interaction between the glycolipid and the TCR (or CD1d, in the case of ceramide modifications) and as such affects their recognition and biological outcome. However, certain 6′-galactose modifications can furthermore induce structural changes in CD1d itself, as demonstrated for the ligand NU-αGalCer (80). The aromatic NU modification is not contacted by the TCR but instead is inserted into the A′ roof, inducing the formation of a small pocket within that roof. It was proposed that the NU group serves as a third anchor in addition to the two alkyl chains that are bound in the A′ and F′ pockets and, as such increases the stability of the CD1d-glycolipid complex, possibly affecting its *in vivo* activity (80).

NKTCR/ligand binding kinetics: the basis for a synaptic duet and synaptic transmission of effector molecules

The kinetic parameters of NKTCR/ligand interaction have been extensively studied. Surface plasmon resonance and tetramer-binding experiments have revealed high-affinity interaction between Vα14i or Vα24i TCR/CD1d αGalCer (or derived analogues): the relative avidity of this interaction is similar to that of high-affinity interactions between the TCR/pMHC complexes (74, 81–85). Interestingly, the half-life of mouse NKTCR/CD1d αGalCer interaction was unusually long (Table S1; references therein). How these kinetic parameters relate to the rapid and robust NKT cell response remains to be elucidated. In this regard, it is interesting to note that an αGalCer analogue, OCH, which has a shortened long-chain sphingosine base (C_9 versus C_{18}) and acyl chain (C_{24} versus C_{26}; Fig 1) and interacts with the Vα14i and Vα24i TCR with lower relative affinity/avidity compared with αGalCer (Table S1; (64, 82, 83), specifically elicits sustained IL-4 with very little IFN-γ response (86). A similar IL-4-biased response is elicited by a diunsaturated (C20-diene) *N*-acyl analogue of αGalCer (Fig. 2; (87) whose binding constant is similar to αGalCer but dissociation rate is similar to OCH (Table S1; (64, 83), the structural basis for which is described below. Hence, the relative TCR binding affinities do not seem to be responsible for the observed T_H1/T_H2 predisposition of structurally related glycolipids. Rather the ability of αGalCer loaded CD1d molecules to accumulate in lipid rafts *in vivo*, in contrast to CD1d molecules that contain OCH or C20-diene appear to influence the cytokine profile (88, 89).

To or not to roof the F′ pocket—It is surprising that despite conserved NKTCR-ligand binding, the equilibrium binding affinity toward microbial glycolipids can vary up to 600-fold compared to αGalCer (Table S1; (72). Essentially two factors have been identified that affect both the association rate of the TCR as well as the dissociation rate. Firstly, the need

to re-orient the galactose of borrelial αGalDAG results in a reduced TCR association (72). Secondly, upon TCR binding onto mouse CD1d-αGalDAG or -αGalACer, the NKTCR induces a structural change in mouse CD1d above the F′ pocket, namely the formation of the F′ roof (72). The F′ roof is already pre-formed upon αGalCer binding to mouse and human CD1d, but not when other known NKT cell agonist bind (Fig 2; (8, 63, 72, 78, 79, 83) and, as such, the TCR does not invest energy into keeping the roof closed upon binding. That results in a more stable complex, indicated by a reduced TCR dissociation rate. The F′ roof is also closed in the previously mentioned ternary complexes of the various αGalCer analogs, as well as in the PtdIno structure (78–80). However, in light of the lack of structures without bound TCR, it is not clear whether the F′ roof is already preformed in those CD1d molecules before TCR engagement. In summary, the agonistic potency of αGalCer and related compounds appears to correlate with the extent to which the F′ roof is pre-formed as well as the ability of the glycolipids to induce further structural changes within CD1d that could enhance CD1d-ligand stability or CD1d-TCR binding stability. Those factors could in turn dictate the biologic outcome upon engaging different ligands, in addition to the pharmacological differences of the glycolipids.

Immune synapse—Conventional T cells and APCs as well as NK cells and target cells form immune synapses in preparation for eliciting an appropriate effector response (90–92). And so do NKT cells and APCs/CD1d-containing planar membrane (93), the specificity of which lies within NKTCR/ligand interactions. Consistent with the kinetic parameters (Table S1), αGalCer and C20-diene efficiently elicit classic immune synapses between NKT cells and the planar membrane by engaging ~10 molecules of CD1d-ligand μm^{-2}. By contrast, OCH forms immune synapses at a tenfold higher concentration (83). Thus, at equal ligand concentration, αGalCer and C20-diene induce very quick and sustained iCa^{2+} flux (a measure of very early T cell activation) when compared with OCH (83).

Conventional T cells polarise certain cytokine receptors (IFN-γR), cytokines (e.g., IFN-γ and IL-2 but not TNF-α and CCL3; (94–96) and lytic granules to the immune synapse (91). αGalCer-pulsed DCs also form immune synapse with freshly isolated NKT cells within 30 min and polarise IFN-γ to the synapse within 50–60 min (93). Similarly, αGalCer and C20-diene rapidly polarised lytic granules to the immune synapse when compared with OCH (83). In this way they engage in synaptic transmission of effector molecules to modulate inflammatory responses to changes in cellular lipid content.

Conclusions

NKT cells localise to portals on microbial entry around cells that express the lipid presenting molecule–CD1d (7, 97, 98). The stability of CD1d-lipid complexes depends on whether the hydrocarbon chain occupying the F′ pocket permits the formation of a roof. NKTCR interfaces its cognate ligand–CD1d-lipid complexes–in a unique mode, which involves germline-encoded ‘hot spots’ that lie within CDR1α, CDR3α and CDR2β loops. The NKT cell-APC synaptic duet is driven by the binding kinetics of NKTCR/ligand interactions. Synapse formation prepares for effector functions and permits synaptic transmission of certain effector cytokines and lytic granules.

Cellular lipid gradients are tightly regulated. Internal and external stressors are known to alter this gradient (52, 99–101). Because CD1d molecules evolved to present lipids, any alterations in the gradient is displayed at the cell surface for an appraisal by NKT cells. By virtue of sensitive ligand recognition (perhaps based on co-operativity; (82), NKT cells can respond quickly to changes in ligand concentration and/or structure. As such, they are known to regulate autoimmune diseases and microbial immunity, and hence inflammation

arising from stressors from within as occurs in autoimmunity or from the outside as in an infection. In this manner, NKT cells can regulate homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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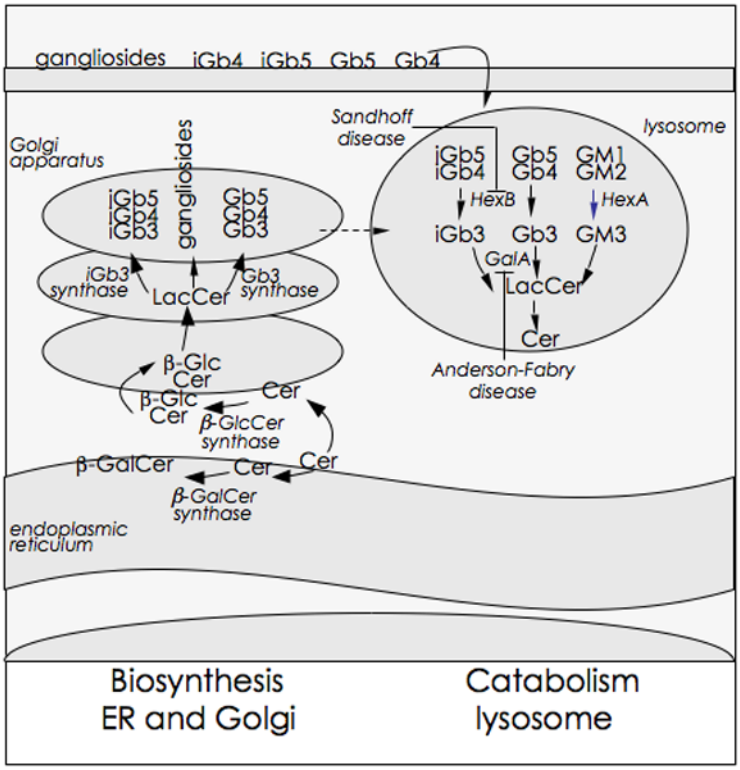

Figure 1. Cellular glycolipid gradients

Glycosphingolipid biosynthesis begins with the synthesis of ceramide on the cytosolic leaflet of the ER. The glycosylation of ceramides results in the formation of glycosphingolipids. The major precursors for mammalian glycosphingolipids are β-glucosylceramide (βGlcCer) and β-galactosylceramide (βGalCer). βGluCer is synthesised by βGlcCer synthase whose catalytic site is predisposed to the cytosolic side of the Golgi apparatus. In contrast, βGalCer is synthesised by an ER luminal enzyme βGalCer synthase. As shown, further glycosylation of βGlcCer results in lactosylceramide (LacCer), gangliosides, globosides and isoglobosides as well as lactoneo- and muco-series of glycolipids (not shown). These distribute to various membranous compartments including the lysosomes. The endogenous iNKT cell antigen iGb_3 is generated by the enzymatic cleavage of β1–3GalNAc from iGb_4 by β-hexosaminidase B (HexB). iGb_3 appear to be rapidly catabolised to LacCer by the action of another lysosomal hydrolase, β-galactosidase A (GalA). Microbes, such as *Salmonella*, and derived products down regulate cellular *GalA* gene expression, which prevents the catabolism of the NKT cell agonist, iGb_3, amongst other glycosphingolipids. Deficiencies in HexB and GalA are know to cause Sandhoff's and Anderson-Fabry diseases, respectively. These lipid storage diseases impact such fundamental processes as macro-autophagy, mitochondrial function as well as protein and lipid trafficking and thereby alter cellular homeostasis. Put together, cellular lipid homeostasis regulates NKT cell function, which in turn can control inflammation.

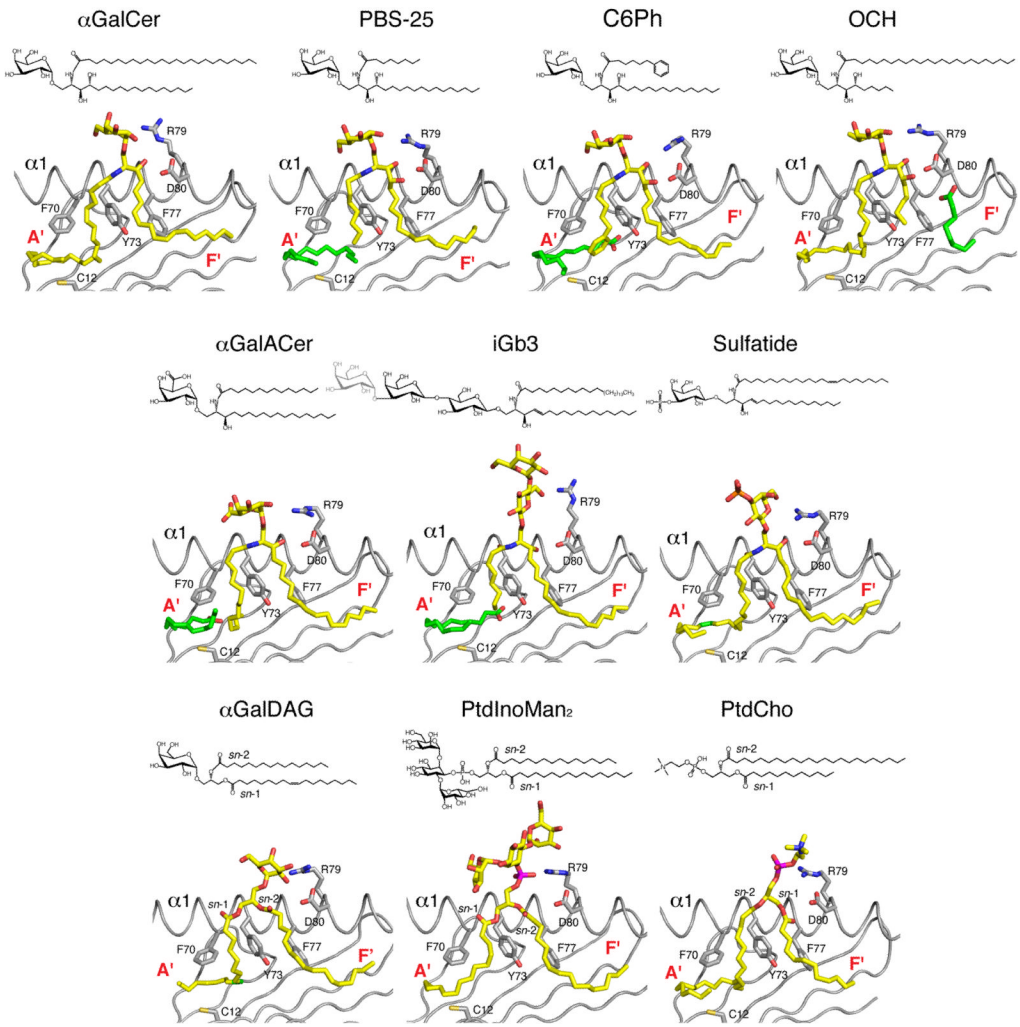

Figure 2. Chemical structure and orientation of ligands in the mCD1d ABG

Top, chemical structures of the ligands; gray, portions not ordered in the corresponding crystal structures. Bottom, a side view of the ABG with the α2 helix removed for clarity. Ligand, yellow; spacer lipids, green; mouse CD1d heavy chain, gray; unsaturations on the acyl chains of the ligands are also green. Some of the residues involved in defining the ABG and contacting the ligand are highlighted. PDB ID: αGalCer (human), 1ZT4;αGalCer, 1Z5L; C6Ph, 3GML; OCH, 3G08; αGalACer, 2FIK; iGb3, 2Q7Y; sulfatide, 2AKR; αGalDAG, 3ILQ; PtdInoMan$_2$, 2GAZ; PtdCho, 1ZHN.

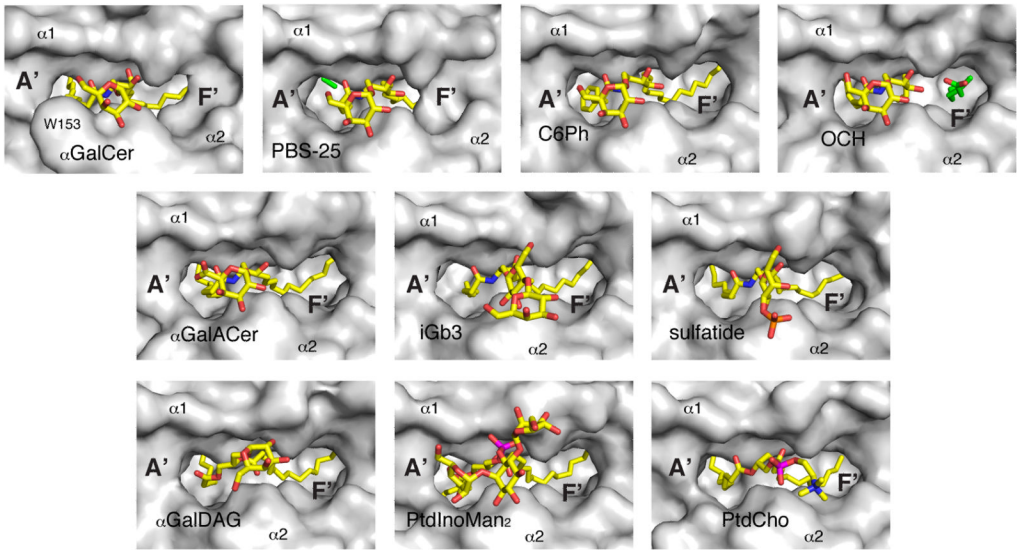

Figure 3. Top view of the CD1d-ligand structures

A top view of the ABG is shown before NKTCR binding with the protein surface in grey and the ligands in yellow. Note the presence of the pre-formed F′ roof exclusively for αGalCer.

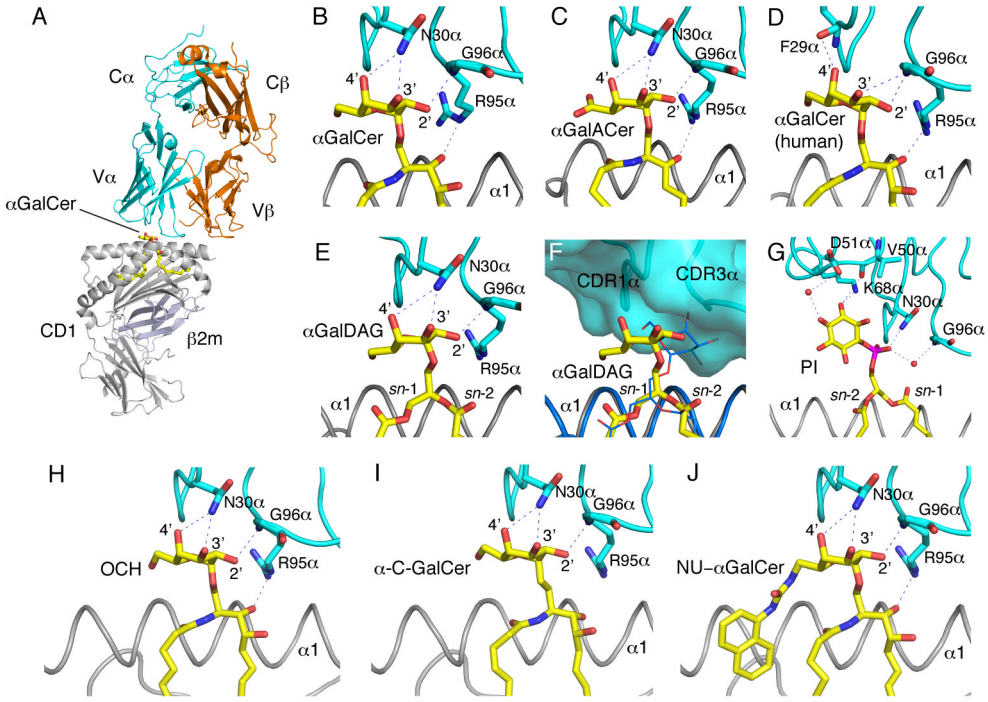

Figure 4. NKTCR/CD1d-lipid interactions

(**A**) Structure of the mouse CD1d-αGalCer-NKTCR complex (3HE6). Mouse CD1d, grey; β2m, light blue; TCR α-chain, cyan; TCRβ-chain, orange; αGalCer, yellow. Note how the contacts between the NKTCR and the ligand or CD1d are dominated by the TCR α-chain. (**B–J**) Conserved interaction of the NKTCR with α-anomeric galactose-containing ligands and with the PtdIno self antigen. The ligands are shown in yellow with CD1d in grey and the TCR α-chain in cyan. Hydrogen bonds between the ligand and conserved residues on the NKTCR are shown as dashed blue lines. (**F**) Superposition of the mouse CD1d-αGalDAG structure before (blue) and after (ligand in yellow; CD1d, gray; TCR α-chain, cyan) TCR binding. Note how a conformational change of the galactose on the ligand is induced upon binding of the NKTCR α-chain in order to avoid steric clashes and allow for the conserved TCR binding footprint onto CD1d. PDB IDs: (**A**–**B**) αGalCer, 3HE6; (**C**) αGalACer, 3O8X; (**D**)αGalCer (human), 3HUJ; (**E**) αGalDAG, 3O9W; (**F**) αGalDAG, 3O9W and 3ILQ; (**G**) PtdIno, 3QI9; (**H**) OCH, 3ARB; (**I**) α-C-GalCer, 3QUX; (**J**) NU-αGalCer, 3QUZ.