Glycolipids as Immune Modulatory Tools

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Abstract: NKT cells are a subset of regulatory lymphocytes characterized by co-expression of the NK cell receptor-CD161 and an invariant TCR-α chain (Vα14-Jα28). They are most abundant in the liver, spleen, and bone marrow. NKT lymphocytes have been implicated in the regulation of autoimmune processes in both mice and humans. Activation of NKT lymphocytes leads to rapid amplification of either IFNγ or IL4, endowing these cells with the capability to mediate both pro-inflammatory and anti-inflammatory immune responses. Activation of this subset of cells is associated with significant liver damage in the Concanavalin A immune mediated hepatitis model. Administration of CD1d ligand has a protective role in collagen-induced arthritis in mice. Disease amelioration was associated with a shift in the immune balance from a pathological Th1 type response towards a protective Th2 type response. In humans, patients with SLE, scleroderma, diabetes, multiple sclerosis, and rheumatoid arthritis have lower numbers of peripheral NKT cells. NKT lymphocytes promote tumor rejection in experimental models of tumor immunotherapy. In contrast, NKT lymphocyte-related anti-tumor activity is associated with pro-inflammatory Th1-type immune responses. NKT cells were shown to have a role in suppression of hepatocellular carcinoma (HCC) *via* immune regulation towards tumor derived antigens, and adoptive transfer of dendritic cells pulsed *ex vivo* with the same antigens.

NKT lymphocytes are activated by interaction of their TCR with glycolipids presented by CD1d, a nonpolymorphic, MHC class I-like molecule expressed by antigen presenting cells, and also by hepatocytes. Several possible ligands for NKT cells have recently been suggested including CD1d bound Glucocerebroside. Glucocerebroside (GC, β-glucosylceramide), a naturally occurring glycolipid, is a metabolic intermediate in the anabolic and catabolic pathways of complex glycosphingolipids. Its synthesis from ceramide is catalyzed by the enzyme glucosylceramide synthase. Inherited deficiency of glucocerebrosidase, a lysosomal hydrolase, results in Gaucher's disease. Patients with Gaucher's disease have altered humoral and cellular immune profiles and increased peripheral blood NKT lymphocytes. CD1d-bound glucocerebroside does not activate NKT cells directly, and may inhibit activation of NKT cells by α-GalCer. On the other hand, glucosylceramide-synthase deficiency was shown to lead to defective ligand presentation by CD1d, with secondary inhibition of NKT cell activation. Recent studies have suggested that a number of glycolipids, including GC, have an immune modulatory effect in several immune mediated disorders. The ability to alter NKT lymphocyte function in various settings and the potential application of natural glycolipids for treatment are discussed.

INTRODUCTION

The innate immune system, inherited from invertebrates, is an ancient immune recognition system of host cells that utilizes germ-line encoded pattern recognition receptors to recognize pathogen associated molecular patterns and trigger a variety of mechanisms directed at pathogen elimination. Cells of the innate immune system include natural killer (NK) cell lymphocytes, monocytes/macrophages, dendritic cells, neutrophils, basophils, eosinophils, tissue mast cells, and epithelial cells. Upon contact with pathogens, macrophages and NK cells may kill pathogens directly or may activate a series of events that both slow the infection and recruit the components of the adaptive immune system, which is based on B and T lymphocytes. In addition to conferring immunity, the innate immune system has been implicated in pathological autoimmunity. Toll like receptors (TLRs), which are classically thought of as sensors of microbial components may have the potential to recognize self-antigens and trigger autoimmune disease [1]. Activation of NK receptors expressed on CD8 T cells may contribute to the development of autoimmune diabetes [2]. The inherent, low-level autoreactivity of certain specialized immune cell types which have both innate and adaptive characteristics, such as CD1d restricted natural killer T (NKT) cells, γδ T cells, and B1 cells, suggests that they may also have the potential to stimulate autoimmunity.

Glycosphingolipids, or glycolipids, are a family of both naturally occurring and synthetic molecules composed of a hydrophobic ceramide backbone (N-acylsphingosine) and a hydrophilic head group made of carbohydrates (mono- or oligosaccharides). They are normally found at the outer surface of cell membranes. The composition of the saccharide moiety is cell type specific, depends on the developmental stage of the organism, and can change with the oncogenic state of a cell. The four principal classes of glycosphingolipids are the cerebrosides, sulfatides, globosides and gangliosides. Enzymatic defects and subsequent accumulation of certain glycolipids lead to known "storage" diseases such as metachromatic leukodystrophy, Gaucher's or Fabry's disease. While the functions of glycolipids are not completely understood, they are believed to play an important role in cell immune recognition and receptor function.

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A. NKT REGULATORY LYMPHOCYTES

Natural killer T (NKT) cells are a subset of regulatory lymphocytes that co-express surface molecules characteristic of both natural killer cells (e.g. C-type lectin NK 1.1) and T lymphocytes [e.g. cluster differentiation 3 (CD3) and α/β T cell receptor (TCR)]. This heterogeneous group can be further divided into at least two subsets: CD4+ and CD4-CD8- (double negative) [3]. Despite this division, many are characterized by their dependence on recognition of the non-polymorphic MHC class I-like CD1d molecule for positive thymic selection and peripheral activation. The largest subset of NKT cells expresses a highly restricted TCR comprised of an invariant TCR α chain with a single rearrangement (in mice Vα14-Jα18, and in humans Vα24-Jα18) coupled with TCR β chains with limited heterogeneity due to marked skewing of Vβ gene usage (mostly Vβ8.2 in mice and Vβ11 in humans) [4]. This population, also referred to as "invariant" NKT cells, (iNKT) is highly conserved in most mammals studied to date.

B. THE ROLE OF NKT CELLS IN IMMUNE RESPONSES

Numerous studies have shown that NKT cells are important regulatory T lymphocytes. They constitutively express cytokine mRNA and within hours of activation can produce large amounts of cytokines such as IFN-γ, TNF, IL-4 and IL-10 [5]. By secreting both Th1 and Th2 associated cytokines they can influence the immune response in a pro or anti-inflammatory direction, subsequently promoting or suppressing the adaptive immune system [6]. Thus, immune responses to certain bacterial, viral, parasitic infections and tumors can be enhanced whereas autoimmune disease and allograft rejection can be suppressed [7]. NKT lymphocytes have been implicated in the regulation of autoimmune processes in both mice and humans [8,9]. Patients with SLE, scleroderma, diabetes, multiple sclerosis, and rheumatoid arthritis have fewer peripheral NKT cells [10-13]. The adoptive transfer of NKT cells has ameliorated disease in several immune-mediated animal models, including experimental autoimmune encephalomyelitis (EAE), immune mediated colitis [14], and graft versus host disease [15]. In addition NKT lymphocytes play an important role in diverse neoplastic and infectious processes, and as such may serve as a target for potential new immune-therapeutic strategies [16].

C. LIGANDS FOR NKT REGULATORY CELLS

Through their semi-invariant TCR, NKT cells recognize glycolipids presented in the context of the CD1d molecule [17]. CD1 proteins are a family of molecules (CD1a-d in humans, CD1d in mice) that have structural homology to major histocompatibility complex (MHC) class I molecules, but are unusual in their ability to present non-peptide lipid-based antigens to T cells [18]. CD1d is highly conserved and homologous in mice and humans, and is expressed by all hematopoietic cells as well as epithelial cells and hepatocytes [19]. The CD1 isoforms have differing affinities for distinct classes of both foreign and self antigens. CD1d and the murine homologue mCD1d present galactosylceramides and hydrophobic peptides. Recent studies have shown that different glycolipids preferentially target to different organelles. As different isoforms of CD1 localize to different subcellular compartments, they allow APCs to present a variety of glycolipid antigens that enter the cell by different pathways and are targeted to different locations [20]. Fig. **1** shows the structures of several ligands for NKT lymphocytes.

D. MICROBIAL CD1D-PRESENTED LIPID ACTIVATORS OF NKT CELLS

A role for NKT cells has been demonstrated in the control of infection by various organisms such as: *Mycobacterium tuberculosis* where they predominate in the anti-mycobacterial granulomatous reaction, [21,22] *Plasmodium bergehi*, *Listeria monocytogenes*, [23] *Ehrlichia muris*, and *Sphingomonas capsulata* [24]. There has been some success in identifying specific microbial glycolipid ligands of CD1d that can activate NKT cells; most notably, α-glycuronosylceramides (α-galacturonosyl and α-glucuronosylceramide, or GSL-1 and GSL-1') derived from the lipopolysaccharide-negative *Sphingomonas* bacteria cell wall [25]. These are of specific significance as they share structural homology with α-galactosylceramide (α-GalCer), a strong agonist of both human and mouse NKT cells. Other examples include the CD1-restricted presentation of *Plasmodium berghei* sporozoite-derived GPI anchor which stimulates NKT-cell-mediated B-cell activation and antibody production, [26] and the phosphatidylinositol tetramannoside (PIM_4) produced by *Mycobacterium bovis* [27]. This suggests a role for NKT cells in the innate response against pathogens which do not activate classical pattern-recognition receptors such as Toll-like receptor 4.

E. α– GLYCOLIPIDS AS NKT LIGANDS

Given the auto-reactivity of the NKT TCR to CD1d, and the limited diversity of TCRs that NKT cells express, it is generally believed that a single, or set of closely related, autologous glycolipid ligands are responsible for the activation of NKT cells. These endogenous ligands have yet to be identified. Recently, the lysosomal glycolipid, isoglobotrihexosylceramide (iGb3) has been proposed as a natural ligand for NKT cells (Fig. **1**). This substance, in its natural or synthetic forms, has the ability to activate most human or mouse NKT cells *in vitro*. Impaired generation of lysosomal iGb3 in mice lacking β-hexosaminidase b resulted in severe NKT cell deficiency, suggesting a role for iGb3 in murine NKT cell development. The combined data suggest that iGb3 or a close structural analogue may be the principal self antigen of NKT cells [28]. However, there still is a lack of direct biochemical evidence for the presence of iGb3 in mice and humans. In addition these results do not rule out the existence of additional endogenous ligands, expressed in various disease conditions or in different cell types.

α-galactosylceramide (α-GalCer or KRN7000) is a synthetic glycolipid that binds with high affinity to CD1d. The α-GalCer–CD1d complex is recognized by both mouse and human T-cell receptors [29]. α-GalCer was originally discovered by the Pharmaceutical Division of the Kirin Brewery Company during a screen for reagents derived from the marine sponge *Agelas mauritianus* that prevent tumour metastases in mice [30]. In most studies a synthetic analogue,

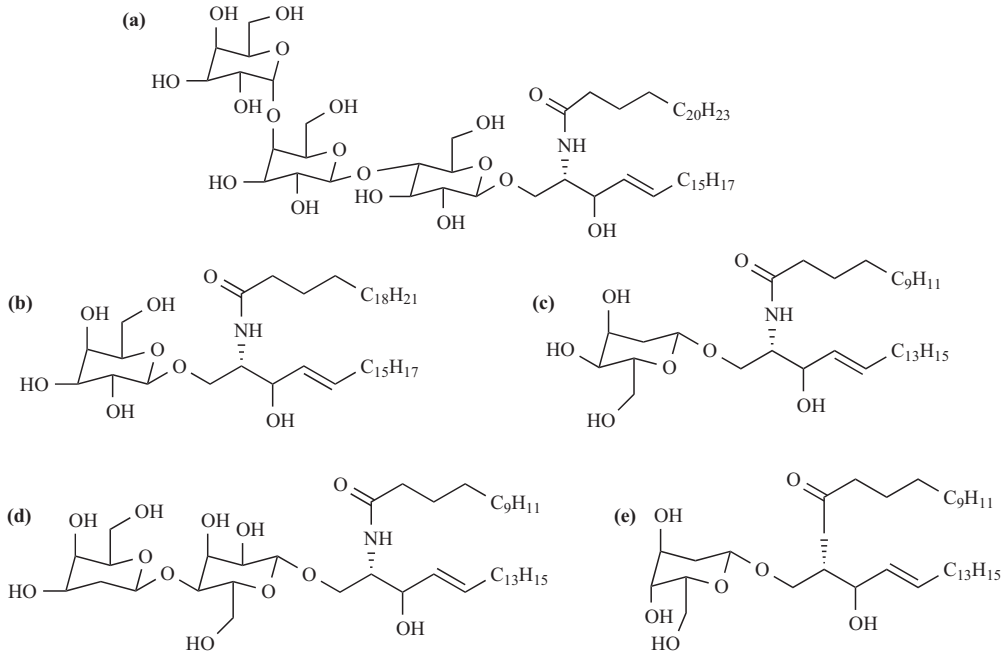

Fig. (1). α and β glycolipids.

(a) isoglobotrihexosylceramide (iGb3). **(b)** α-galactosylceramide (α-GalCer). **(c)** β-glucosylceramide (GC). **(d)** β-Lacotsylceramide (LC). **(e)** β-Galactosylceramide (GLC).

KRN7000, of this natural product has been used and is usually still referred to as α-GalCer. Interestingly, reactivity with α-GalCer is not restricted to NKT cells from mice but also includes NKT cells from humans, macaques and rats. α-GalCer is a glycosphingolipid, a chemical category that includes many endogenous glycolipids. It is distinguished, however, by the stereochemistry of the bond that joins the asymmetric 1′ carbon of the sugar to the lipid. In nearly all natural cases, this bond is in the β-anomeric form, but the sponge-derived glycosphingolipid has an α-linkage. It is assumed that α-GalCer only mimics the natural ligand that NKT cells recognize. The crystal structures of human and murine CD1 molecules with and without α-GalCer have been determined, showing that while the ceramide tail remains hidden in the CD1d cleft, the galactosyl head is exposed for TCR recognition [31]. α-GalCer binds to CD1d with one of the highest affinities of the known ligands.

F. IMMUNE MODULATORY ACTIVITIES OF A-GALCER AND ITS ANALOGS

Activation of NKT cells *via* α-GalCer has been shown to affect numerous models of malignancy, infection and autoimmune disease [32]. In models with strong NKT cell involvement such as in type I diabetes-prone NOD mice, activation of NKT cells with α-GalCer delayed disease induction and prevented its recurrence [33,34]. On the other hand, in disease models where NKT cells play a "pathogenic" role such as in the F1 mouse model of lupus nephritis (NZB x NZW) [35] or the apolipoprotein E knockout mouse model of atherosclerosis, [36,37] NKT cell activation by α-GalCer led to disease aggravation.

It has been suggested that the length of the glycolipid sphingosine chain can affect cytokine release by activated NKT cells [38]. In an attempt to elucidate this effect various analogs of α-GalCer with different chain lengths were studied [39]. OCH is a truncated analog of α-GalCer, where the sphingosine chain has been shortened from 18 to 9 carbons. Following administration of OCH to mice the early production of IL-4 by NKT cells remained intact while the bulk of IFN-γ, mostly derived from NK cells was lost, leading to a Th-2 biased response. Additional analogs show that progressive truncation of either the fatty acyl chain or the sphingosine base of α-GalCer leads to a Th2-biased cytokine profile. It has been shown that the ratio of IL-4 to IFN-γ released by NKT cells is influenced by the length of the lipid chain, with shorter chain lengths increasing the ratio [39]. However, loss of too much lipid resulted in the complex with CD1d being too unstable to interact well with NKT cell receptors. This is demonstrated by the finding that the OCH/CD1d tetramer complexes preferentially bind to Vβ8.2-bearing murine NKT cells, while α-GalCer/CD1d tetramers bind all NKT cells with high avidity. In addition OCH/CD1d complexes demonstrate a shorter half life in comparison to α-GalCer/CD1d complexes. *In vitro* dose/response experiments with mouse splenocytes indicate that OCH is a less potent inducer of IL-4, IL-2, and total cell proliferation than α-GalCer [40]. C20:2 is an α-GalCer analogue that contains a C20 fatty acid with *cis*-unsaturations at positions 11 and 14. Although similar to OCH in eliciting Th2-biased responses, it induces IL-4 and IL-2 levels more comparable to those stimulated by α-GalCer [41]. At the opposite end of the spectrum, the C-glycoside of α-GalCer (α-C-GalCer) differs by having a CH2 group in place of the O-linkage between the sugar moi-

ety and the sphingosine base. The increased stability of the C-linkage possibly gives α-C-GalCer a longer half-life *in vivo*. Administration of α-C-GalCer leads to a strong Th-1 biased response with sustained IFN-γ levels for several days compared to the 24 hours induced by α-GalCer [42]. Treatment with OCH was more efficacious than α-GalCer in the Th-1 mediated autoimmune disease models of encephalomyelitis and colitis [43]. Treatment with α-C-GalCer was more potent than α-GalCer in mouse models of malaria and malignant tumors. Despite their promising effects in diverse disease situations, the clinical use of α- glycolipids has been limited by their side effects, mainly hepatotoxicity [44].

G. β-GLYCOLIPIDS AS POSSIBLE NKT LIGANDS

Beta glycolipids are naturally occuring intermdiates in the anabolic and catabolic pathways of complex glycosphingolipids, and are found in cell membranes. Studies in the past suggested that beta glycolipids have no effect on NKT cells [45]. Recent data has suggested that these compounds may have an important NKT cell mediated immune modulatory effect. Glucocerebroside (β-glucosylceramide, GC, Fig. **1**), a beta glycolipid, is degraded into ceramide by glucocerebrosidase. Inherited deficiency of glucocerebrosidase, a lysosomal hydrolase, results in Gaucher's disease [46]. Patients with Gaucher's disease have altered humoral and cellular immune profiles and increased peripheral blood NKT lymphocytes [47,48]. CD1d-bound GC does not activate NKT cells directly, and may inhibit activation of NKT cells by α-GalCer. On the other hand, glucosylceramide-synthase deficiency was shown to lead to defective ligand presentation by CD1d, with secondary inhibition of NKT cell activation [49]. *In vitro*, administration of GC led to a 42% decrease in NKT cell proliferation in the presence of DCs, but not in their absence [50].

Additional naturally occuring beta glycolipids such as: β-Lacotsylceramide (LC) and β-Galactosylceramide (GLC) are being tested for their immune-modulatory effects.

Administration of β-glycolipids in several Th1 mediated disease models such as hepatitis, colitis, metabolic syndrome and acute GVHD, alleviated the disease while inducing a Th2 cytokine profile. In a murine model of concanavalin A induced hepatitis, administration of GC led to significant amelioration of liver damage. The beneficial effect was associated with a 20% decrease in intrahepatic NKT lymphocytes, a significant lowering of serum IFN-γ levels and decreased STAT-1 and STAT-6 expression [51]. Similar results were obtained with additional β-glycolipids such as LC and GLC (unpublished data). The administration of GC to leptin-deficient ob/ob significantly improved metabolic alterations in these mice [52]. Liver fat content was reduced significantly in both MRI and histological examinations. In addition treated mice achieved near-normalization of glucose tolerance and decreased serum triglyceride levels. These effects have been associated with a marked increase of the peripheral/intrahepatic NKT cell ratio. Similar results where obtained in sand rats (unpublished data). In a murine model of experimental colitis (2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis) GC, LC and GLC led to varying degrees of disease amelioration associated with a Th2 cytokine shift [53]. Finally in a semi-allogeneic model of acute graft-versus-host disease (GVHD) GC-treated mice manifested a significant decrease in skin, bowel, and liver GVHD manifestations. The beneficial effect of GC was associated with decreased IFNγ and increased serum IL4 levels, as well as a significant increase in the intrahepatic to peripheral NKT lymphocyte ratio and in intrahepatic CD8+ lymphocyte trapping [54].

In contrast, in Th2 mediated models of disease, administration of β-glycolipids also led to NKT mediated disease alleviation associated with an opposite Th1 immune shift. In a murine model of B16 melanoma a marked suppression of tumor volume and lung metastases were noted (unpublished data). Similarly, in a Hep3b model of hepatocellular carcinoma GC led to improved survival rates and a decreased tumor volume [55]. These effects have been associated with an 11 fold increase in intrahepatic NKT lymphocyte number**.**

Taken together, these results suggest that certain β-glycolipids may serve as a "fine tuners" for NKT lymphocyte-mediated immune responses and may have a beneficial effect in seemingly opposing disease models.

In summary, NKT lymphocytes have been implicated in the regulation of autoimmune processes in both mice and humans. Glycolipids hold promise as new immune modulatory agents, with a possible role in the treatment of immune-mediated disorders. The ability to alter NKT lymphocyte function in various setting using different ligands, may make them possible immune modulatory agents in the future.

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