Glycolipids for natural killer T cells

Paul B. Savage,*[a] Luc Teyton[c] and Albert Bendelac[b]

Received 18th April 2006
First published as an Advance Article on the web 9th June 2006
DOI: 10.1039/b510638a

Natural killer T cells (NKT cells) play a central role in regulating immune responses influencing conditions ranging from autoimmune to infectious diseases. NKT cell responses are induced by recognition of glycolipid antigens presented by CD1d, an antigen presentation protein. In the last 10 years great strides have been made in understanding the types of glycolipids recognized by NKT cells. These advances have included determination of the lipid and carbohydrate recognition requirements for stimulation and identification of ''natural'' antigens for these cells.

[a]*Reed M. Izatt Professor of Chemistry and Biochemistry, Associate Chair, Department of Chemistry and Biochemistry, Brigham Young University, C100 BNSN, Provo, UT 84602, USA. E-mail: paul_savage@byu.edu; Fax: 801-422-0153; Tel: 801-422-4020*
[b]*University of Chicago, Chicago, IL 60637, USA*
[c]*Scripps Research Institute, La Jolla, CA 92037, USA*

Paul B. Savage

Paul B. Savage received his PhD in 1993 from the University of Wisconsin and was an NIH postdoctoral fellow at the Ohio State University. He began work as a principal investigator at Brigham Young University in 1995. He is now the Reed M. Izatt Professor of Chemistry and Biochemistry. Research foci are the study of the interactions of compounds with bacterial glycolipids and the design and synthesis of glycolipids that stimulate responses from natural killer T cells.

Luc Teyton

Luc Teyton received an MD degree in 1980 and a PhD in Immunology in 1987 from Paris University. He completed postdoctoral work at The Scripps Research Institute, where he worked with Per Peterson on antigen presentation and the determination of the dual role of the invariant chain. As an assistant professor at TSRI he pioneered work on the expression of recombinant TCR and MHC molecules. This work was capped by the determination of the first TCR and TCR–MHC complex structures while he was employed by Johnson & Johnson. Back at TSRI since 1997, his work has been focused on the association MHClautoimmunity, the molecular switches of T cell activation and the biology of CD1 molecules and the presentation of lipids to the immune system.

1 Introduction

1.1 Glycolipid recognition by NKT cells mediated through association with CD1d

Natural killer T (NKT) cells are a subset of T cells and play a central role in regulating immune responses (for recent reviews of the immunology of NKT cells, see refs 1–4). Stimulation of NKT cells leads to release of signaling peptides, termed cytokines and chemokines, that are recognized by other cells of the immune system. NKT cell-mediated regulation of immune responses has been demonstrated to influence a large number of disease states (reviewed in ref. 4). Diminished numbers of NKT cells have been correlated to an increased incidence of autoimmune diseases including type 1 diabetes and rheumatoid arthritis. Stimulation of NKT cells (*vide infra*) can positively or negatively influence forms of lupus and generates positive outcomes from viral, bacterial and parasitic infections. Finally, some forms of human cancer have been correlated with a loss of NKT cells, and in animal models stimulation of NKT cells leads to decreases in tumor size and growth. Due to the broad impact that NKT cells play on health, there has been intense interest in understanding how NKT cells are stimulated and the extent to which NKT cell responses can be controlled.

Albert Bendelac

Albert Bendelac received his MD in 1985 and PhD in 1992 at the Universite Paris VI and Paris VII, France. He completed his postdoctoral work at the National Institutes of Health, Bethesda, MD before joining the faculty at Princeton University in 1994. He is currently a Howard Hughes Medical Institute Investigator and Professor in the Department of Pathology at the University of Chicago. His main research work focuses on NKT lymphocytes and the biology of lipid antigen presentation.

NKT cells can be divided into multiple subsets, and the best-studied and most populous subset includes those cells that present a T cell receptor (TCR) made up of the Va14 subunit (for those made up of other subunits see refs 5 and 6) in mice and the Va24 subunit in humans. NKT cells expressing the Vα14/Vα24 subunit will be the focus of this review. Antigen presenting cells expressing the membrane-anchored protein CD1d present glycolipids that are recognized by TCRs on NKT cells. CD1d is one of a family of glycolipid presentation proteins that are evolutionarily related to antigen-presentation proteins known to present peptides (major histocompatibility complexes). Recent structural studies have demonstrated how this protein is exquisitely tuned to present glycolipid antigens.[7–9] Lipid chains are bound in "tunnels" in the interior of the protein, and sugar head groups are presented to the specialized set of TCRs on NKT cells.

Through work by multiple research groups, it has been established that NKT cell stimulation is mediated by presentation of specific classes of glycolipids by CD1d to NKT cells. Many of the advances in the field have come from studies of NKT cell responses to a glycolipid termed KRN7000 (Fig. 1), which was developed from structure–activity studies of the anti-tumor properties of glycolipids of marine origin (*vide infra*).[10–15] While excellent reviews have recently been published describing the immunological role of NKT cells and the potential for use of NKT cells responses for intervention in human disease,[1–4] this review will focus on the nature of the glycolipids that stimulate NKT cells and the influences of glycolipid structure on NKT cell responses.

1.2 Cytokine release by NKT cells

Upon stimulation, NKT cells can rapidly release a variety of cytokines, and these cytokines can promote two different types of immune response. One group of cytokines, including interferon-γ (IFN-γ) and interleukin-2 (IL-2), cause an inflammatory response, termed a T helper 1 (Th1) response. In contrast, other cytokines that can be released by NKT cells include IL-4 and IL-10, and these cytokines result in an immunomodulatory or a Th2 response. Proinflammatory Th1 responses are effective in controlling bacterial, parasitic, and viral infections and can result in immune responses to tumors. However, autoimmune diseases including multiple sclerosis, lupus, rheumatoid arthritis and type 1 diabetes are Th1-mediated. Th2 cytokines can antagonize the immunostimulatory properties of Th1 cytokines; consequently, production of Th2 cytokines can ameliorate autoimmune diseases.

A puzzling aspect of NKT cell responses is that both Th1 and Th2 cytokines can be produced in response to stimulation.[16] For example, in an animal model of type 1 diabetes, when IL-4 was prevented from being produced, administration of KRN7000 caused rapid onset of the disease.[17] In contrast, when production of IFN-γ was restricted, KRN7000 delayed the onset of the disease. When production of both cytokines was unrestricted, administration of KRN7000 had no significant effect on progression of the disease; in effect the Th1 and Th2 responses had canceled one another. Potential medicinal use of NKT cell responses will require control over the types of response generated: for treating infection and tumors, a Th1 response is desirable, and a Th2 response is appropriate for prevention or inhibition of the progression of autoimmune diseases.

1.3 Means of studying NKT cell responses

Both *in vitro* and *in vivo* methods have been developed for the study of NKT cell responses to glycolipid presentation by CD1d. NKT cells are a subpopulation of T cells and in the spleen, where NKT cell populations are relatively high, they represent less than one percent of the total cells. Cells from the spleen contain not only NKT cells but also antigen-presenting cells (APCs), which express CD1d. Thus, the population of cells from the spleen contains all of the cells necessary for sequestering glycolipids, loading them into CD1d, and presenting them to NKT cells. Cytokine release is monitored using ELISA. A drawback of using splenocytes is that cells involved in other aspects of the immune system are present; consequently, responses are not limited to NKT cells and may come from recognition of antigens by other cells (*e.g.*, recognition of lipopolysaccharide by monocytes). A simpler system involves use of NKT cell hybridomas and APCs or CD1d adsorbed onto plastic plates. The advantage of using this system is that it is CD1d–NKT cell restricted. *In vivo* responses are typically measured by injecting the glycolipid (intraperitoneal or intravenous) into mice and monitoring plasma cytokine levels using ELISA.

1.4 Therapeutic potential for NKT-cell stimulating glycolipids

In multiple animal models of human disease, the therapeutic potential of harnessing NKT cell responses has been demonstrated. Initial structure activity studies with NKT-cell stimulatory glycolipids were conducted in the context of anti-tumor properties.[10] In addition, anti-bacterial, anti-parasite, and anti-viral effects of NKT cell stimulation have been well documented.[4] In addition and at times in contradiction, NKT cell responses to glycolipids have proven to improve the outcomes from animal models of autoimmune diseases.[1–4,16] Because NKT cell Th1 and Th2 responses can offset one another, means of polarizing cytokine release toward *either* Th1 *or* Th2 may be important in therapeutic use of NKT cell responses. In the context of stimulatory glycolipids, an understanding of how structure influences

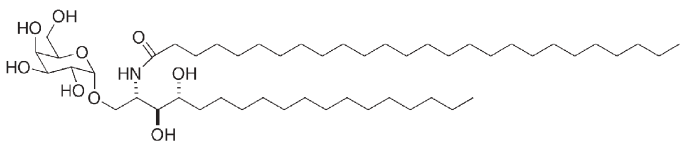

Fig. 1 Structure of glycolipid antigen KRN7000.

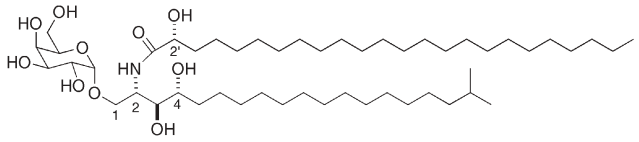

Fig. 2 Structure of agelasphin 9b.

cytokine release profiles is essential. An additional key point is that CD1d-mediated glycolipid presentation to NKT cells evolved as an important aspect of immune regulation, and this system must be present to survey for specific "natural" antigens (KRN7000 is generally not considered to be a natural antigen). Therefore, immunoregulation by NKT cells requires an understanding of glycolipid antigens likely to be present *in vivo*.

2 α-Glycosylceramides as NKT cell stimulating agents

2.1 Discovery of α-galactosylceramides as immunostimulants

Screening of marine natural products for anti-tumor activities led Koezuka and coworkers[14,15] at Kirin Brewery to the discovery of glycolipids, termed agelasphins (for an example see Fig. 2), isolated from the sponge *Agelas mauritianus*. This discovery, reported in 1993, was notable because the galactosylceramides contained α-glycosidic bonds between the sugar and ceramide, and in general glycosylceramides found in higher organisms only have β-glycosidic linkages. An understanding of the mechanism of immunostimulation had not been developed at the time although it was realized that the agelasphins caused lymphocyte proliferation. Initial structure–activity studies by the Kirin group focused on the ceramide portion of the molecule.[10] These studies indicated that the hydroxyl group at C2′ (see Fig. 2 for ceramide numbering) does not significantly influence anti-tumor activity, that the C4 hydroxyl group plays a minor role, and that the hydroxyl group at C3 is necessary to maximize activity. They also found that the phytosphingosine chain could be simplified, and a straight-chain lipid was substituted for the branched. Extending the acyl chain length in agelasphin 9b by two carbons yielded KRN7000.

As the immunoregulatory role that NKT cells played was elucidated, KRN7000 became the principal glycolipid used in the study on NKT cell stimulation. As described above, KRN7000 stimulates production of both Th1 and Th2-related cytokines by NKT cells, although at the time of its development the nature of the immune responses to the agelasphins and compounds used in the studies were not fully understood.

2.2 Comparisons of modifications to sugar head groups

A second structure–activity study conducted by Taniguchi and coworkers[18] focused on the carbohydrate portion of KRN7000 with a readout of NKT cell proliferation. The following requirements for NKT cell stimulation were determined (summarized in Fig. 3): α-galactosylceramide (KRN7000) is more active than α-glucosylceramide; mannosylceramide is inactive; and β-galactosylceramide is inactive. A study of the affinity of CD1d loaded with various glycolipids for a soluble form of the TCR was recently reported that shed light on NKT cell responses to monoglycosylceramides.[19] The dissociation constant of the TCR for CD1d loaded with KRN7000 was determined to be 0.35 μM. Affinity for the CD1d-α-glucosylceramide complex was *ca*. 10-fold lower, while the K_D for CD1d-α-mannosylceramide complex was 13.23 μM. Binding was not detected when KRN7000 was substituted by β-galactosylceramide. These results demonstrate that even relatively minor changes in glycolipid structure can result in large affinity differences among binding partners. Recognition may come from glycolipid interactions with CD1d and/or the TCR. If glycolipids are similarly bound by CD1d, it is most likely that recognition occurs concomitant with TCR binding. However, glycolipid binding by CD1d presumably positions the glycolipid in a position in which it can be recognized by the TCR. Consequently, if the glycolipid is not "properly" presented by CD1d, it may not be effectively recognized by the TCR.

Taniguchi[18] and coworkers also determined that α-galactosylceramide substituted with sugars at the C2, C3 or C6 position of the galactose, giving **1**, **2** and **4** in Fig. 4, stimulated nearly as well as KRN7000. Later, Kronenberg and coworkers[20] showed that disaccharides **1** and **2** must be truncated by APCs to the corresponding α-galactosylceramide for presentation and stimulation of NKT cells. Zhou, *et al.*,[21] demonstrated that glycolipids **3a** and **3b** must also be processed to α-galactosylceramide to allow stimulation. In contrast, 1–6 linked disaccharide **4** (Fig. 4) stimulates NKT cells without truncation to the monoglycosylceramide. These results suggest that the glycolipid–CD1d–TCR interaction tolerates a small molecule at the C6 position on the sugar in α-galactosylceramide but that binding of CD1d or the TCR to glycolipids is inhibited by other substitution patterns. Recently, the crystal structure of CD1d complexes of KRN7000 and a related glycolipid were reported,[7,8] and from these structures it is clear that disaccharides **1** and **2** would not be able to bind to CD1d in a manner similar to KNR7000 (*i.e.*, the second sugars would interfere with the hydrogen-bonding pattern observed with KRN7000) (see Fig. 5). From the structures, it is less clear why substitution at the C4 position sugar in α-galactosylceramides **3a** and **3b** inhibits stimulation

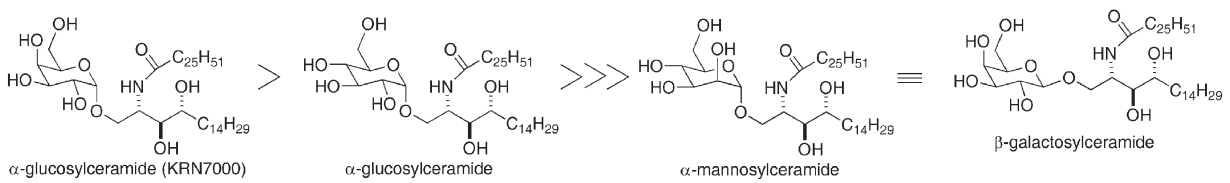

Fig. 3 Structures of monoglycosylceramides and comparison of their propensity to stimulate proliferation of NKT cells.

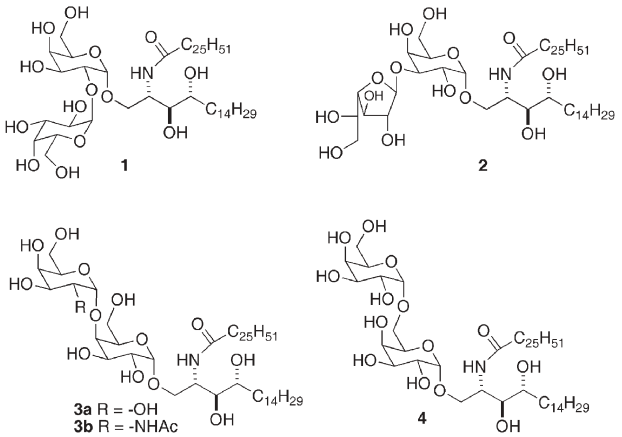

Fig. 4 Structure of diglycosylceramides used in the study of the requirements for processing of diglycosylceramides for stimulation of NKT cells. Only **4** is capable of stimulating NKT cells without truncation to the monoglycosylceramide.

of NKT cells. It is possible that CD1d binding is not hampered but that interactions with the TCR are interrupted by the additional sugar.

The observation that a small molecule at C6″ on α-galactosylceramide was well tolerated in CD1d and TCR interactions led Zhou, *et al.*[22] to a study in which a variety of small molecules were appended at this position yielding compounds **5–7** (Fig. 6) with small fluorophores as well as biotin attached to the glycolipid through an amide bond. Studies of cytokine production of NKT cells in response to these glycolipids revealed that the small fluorophores and biotin were also well tolerated. That is, **5–7** stimulated NKT cells comparably to

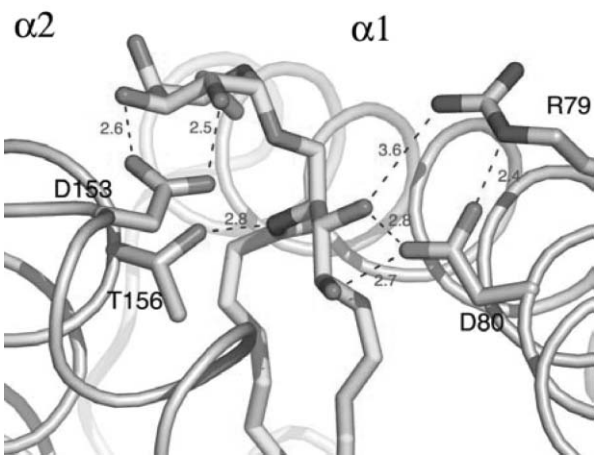

Fig. 5 Representation of the hydrogen-bond network between CD1d and an α-galactosylceramide ligand observed in the CD1d–glycolipid crystal structure.[7] The lipid chains are pointing into binding grooves with the galactose between the α1 and α2 helices. Asp153 engages in hydrogen bonds to the C2 and C3 hydroxyl groups, while Asp80 and Arg79 on the α1-helix stabilize the phytosphingosine backbone. Hydrogen bonds are depicted as dashed lines and distances (Å) between the hydrogen-bonding partners are labeled accordingly. (Reproduced by permission from ref. 7. Copyright 2006 Nature Publishing Group.)

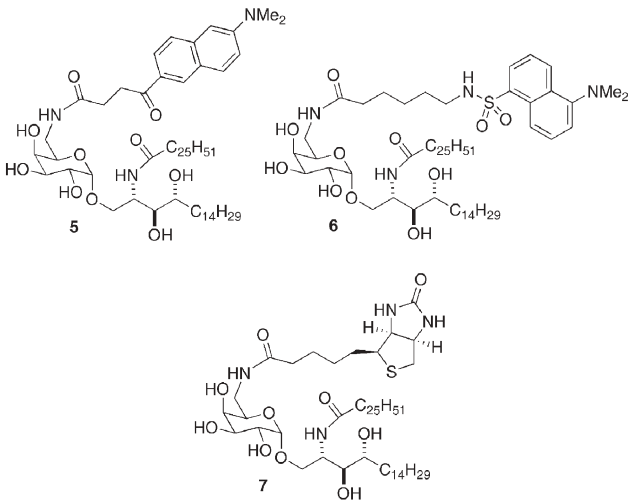

Fig. 6 Structures of C6″ appended α-galactosylceramides.

KRN7000. These compounds are now being used to study glycolipid trafficking and CD1d loading.

2.3 Effects of ceramide modifications

The early studies by Koezuka and coworkers demonstrated structural requirements of ceramides necessary for anti-tumor activity of α-galactosylceramides.[10] These requirements included: hydroxyl groups at C3 and C4 and a relatively long acyl chain. Since this initial report, careful studies of the influence of ceramide structure on NKT cell stimulation and on glycolipid–CD1d–TCR interactions have been reported that more clearly describe how structure effects NKT cell responses. These studies can be separated into two classes: the first includes studies of effects of variations in the polar portion of ceramide, and the second includes work with modifications to the lipids in ceramide.

Recently, Sidobre, *et al.*,[19] reported a comparison of the affinities of a solubilized form of the TCR from NKT cells for CD1d loaded with the glycosylceramides shown in Fig. 7. It is striking that elimination of hydroxyl groups on the ceramide group results in a loss of affinity considering the fact that this portion of the molecule is unlikely to make contact with the TCR. As discussed above, the hydroxyl groups at C3 and C4 may facilitate orientation of the sugar head group by CD1d for recognition by the TCR. However, as demonstrated by this and a further study,[23] the hydroxyl group at C3 is much more important than that at C4 for orienting the glycolipid in CD1d.

A key question in understanding glycolipid–NKT cell interactions is whether glycolipid structure influences not only the magnitude of NKT cell stimulation but also the nature of the stimulation. Because NKT cells can produce cytokines that result in conflicting responses, the possibility exists that glycolipid structure can result in a polarization of NKT cell responses toward either a Th1 or a Th2 response. In 2001, Miyamoto, *et al.*[24] reported that truncation of the phytosphingosine chain of KRN7000 yielded a glycolipid, termed OCH (Fig. 8), that changed the response of NKT cells relative to KRN7000. Whereas stimulation of NKT cells with KRN7000 results in release of a mixture of Th1 and Th2

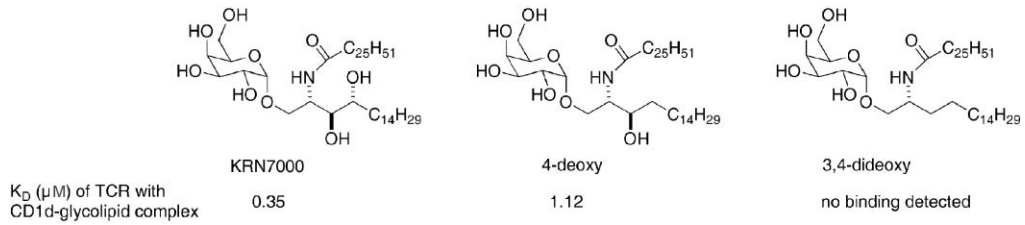

Fig. 7 Structures of glycolipids used in a study of the binding of CD1d–glycolipid complexes to a TCR from NKT cells. Dissociation constants are given for the ternary complexes.

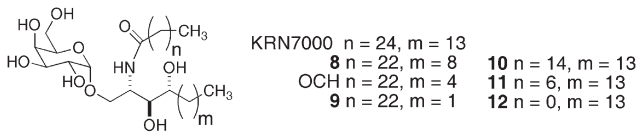

Fig. 8 Structures of lipid chain-truncated glycolipids.

cytokines, stimulation with OCH results in release of primarily Th2 cytokines. The Th1/Th2 bias was quantified by measuring relative amounts of IFN-γ, a Th1 cytokine, and IL-4, a Th2 cytokine. In further studies it was demonstrated that administration of OCH ameliorated symptoms in multiple Th1-mediated autoimmune diseases including experimental autoimmune encephalomyelitis (a model of human multiple sclerosis),[24] collagen-induced arthritis,[25] and type I diabetes.[26] In the study by Miyamoto *et al.* only one chain-truncated glycolipid is reported. Goff, *et al.*[27] reported the synthesis and NKT cell stimulating properties of a series of α-galactosylceramides (Fig. 8) in which the phytosphingosine or acyl chain lengths of KRN7000 were varied. These studies demonstrated that truncation of either the phytosphingosine or acyl chain resulted in glycolipids that biased NKT cells toward release of Th2 cytokines. That is, OCH and **11** similarly biased NKT cell responses toward IL-4 release over IFN-γ. Further truncation of either lipid chain, yielding **9** and **12**, gave compounds that only weakly stimulated any response from NKT cells.

The lipid portions of glycolipids are sequestered in tunnels in CD1d; consequently, it is not immediately apparent how lipid chain length influences NKT cell responses. It has been proposed that the glycolipid–CD1d–TCR complexes containing OCH are less stable than those with KRN7000, which results in a less prolonged NKT cell stimulation.[28] Teyton and coworkers[7] reported the crystal structure of the **11**–CD1d complex. In the complex, the relatively short acyl chain of **11** only partially fills the lipid-binding tunnel in the protein (Fig. 9). The remainder of the tunnel is filled by a "spacer lipid." Because **11** is able to bind above this spacer lipid, it loads very efficiently into CD1d, whereas a glycolipid with a longer acyl chain (*e.g.*, KRN7000) would require displacement of the spacer lipid. In the report by Teyton and coworkers, it was demonstrated that the cell types that present **11** are different from those that present KNR7000 (primarily B cells *vs.* primarily dendritic cells), an idea first proposed by Joyce, *et al.*[29] This difference is proposed as the origin of the distinct NKT cell responses to these two glycolipids.

In further work on the effects of altering acyl chain characteristics in KRN7000 and the effects on NKT cell responses, Porcelli and coworkers[30] reported a series of glycolipids that incorporated varied amounts of unsaturation (*i.e.*, *cis* double bonds) into the acyl chain of KRN7000. α-Galactosylceramides with 1–4 double bonds in the acyl chain were prepared, and similar to the lipid chain-truncated glycolipids, those with *cis* double bonds in the acyl chain were also reported to cause a Th2 bias in NKT cell responses. The α-galactosylceramide identified as having the most attractive Th2 biasing properties contained a C20:2 acyl group (derived from 11,14-eicosadienoic acid).

As demonstrated by the truncation of diglycosylceramides **1–3** (Fig. 4) to KRN7000, glycolipids are exposed to glycosidases before presentation to NKT cells. Considering this fact, Franck, Tsuji, and coworkers[31,32] prepared and tested

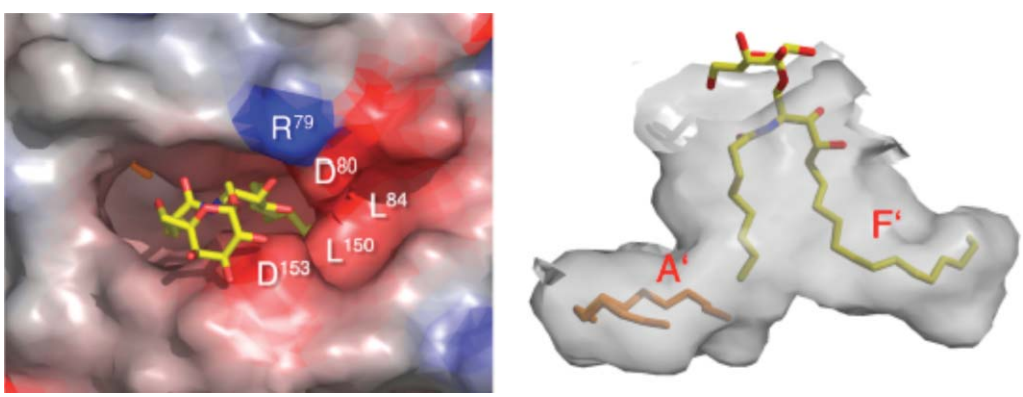

Fig. 9 Structure of the CD1d–**11** complex. Molecular surfaces are either shown with electrostatic potentials (left) in a top view or with transparent binding pockets. Amino acid residues that are involved in hydrogen bonding or the formation of the roof above the F′ pocket are labeled along with the A′ and F′ lipid binding tunnels. (Reproduced with permission from ref. 7. Copyright 2006 Nature Publishing Group.)

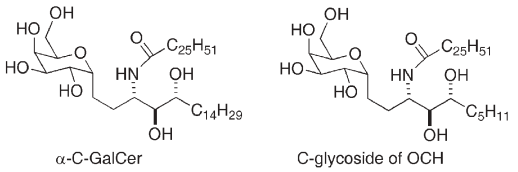

Fig. 10 Structures of *C*-glycosides related to KRN7000 and OCH.

an α-galactosylceramide in which the glycosidic oxygen was replaced by a methylene group (Fig. 10). They found that this glycolipid, termed α-C-GalCer, stimulated very strong Th1 responses *in vivo* from NKT cells. This response was as much as 1,000 times stronger that that induced by KRN7000. For example in a mouse model of malaria, responses to 1 ng of α-C-GalCer effectively cleared sporozoites from the liver, while a dose of *ca*. 1 μg of KRN7000 was required for comparable activity. It is hypothesized that the greater activity of α-C-GalCer is due to its greater stability. Recently, synthesis of the *C*-glycoside version of OCH (Fig. 10) was reported,[33] but NKT stimulating properties of this glycolipid have not been reported. Replacement of the glycosidic oxygen in the context of KRN7000 resulted in a compound with a greater Th1 bias; consequently, it will be interesting to find if the cytokine release profile of OCH is altered towards Th1 cytokines by formation of the *C*-glycoside.

3 Natural antigens for NKT cells

The glycolipids that were first identified as ligands for NKT cells were isolated from marine sponges, and this fact has led to the assumption that these glycolipids are not natural antigens for NKT cells. As well stated in a recent review of the immunology of NKT cells: "The true identity of antigens presented to CD1d was long unknown because it seemed unlikely that this system would have been conserved evolutionarily as a defence mechanism against marine sponges."[34] Consequently, there has been great interest in identifying antigens to which humans (and other mammals that produce NKT cells) would be exposed. These natural antigens could be separated into two groups: (1) antigens that are produced by the host (endogenous antigens); and (2) antigens from foreign pathogens (exogenous antigens). The strongest evidence for the presence of an endogenous antigen is that positive selection of NKT cells in the thymus requires presentation of an antigen recognized by the TCR. The best evidence for the presence of exogenous antigens is that antigen presentation proteins related to CD1d have been characterized as presenters of microbial glycolipids, and it was speculated that NKT cells might survey for the presence of infectious agents.

3.1 Discovery of iGb3 as an endogenous antigen

A key aspect of the search for the endogenous antigen was based on the idea that mice lacking the endogenous antigen would also lack NKT cells. Without positive selection employing the natural antigen in the thymus, NKT cells undergo apoptosis and their numbers are severely reduced or they are eliminated entirely. To gain clues about the types of glycolipids that might be natural antigens, mice with glycolipid processing deficiencies were surveyed for the presence of NKT cells.

In 2003, Joyce and coworkers[35] reported that a cell line lacking the enzyme β-D-glucosylceramide synthase was unable to stimulate NKT cells presumably due to a lack of the endogenous antigen (Fig. 11). Notably, mice lacking β-galactosylceramide produce NKT cells in a fashion similar to wild-type mice. The work by Joyce and coworkers suggested that the endogenous antigen might be derived from β-glucosylceramide, but its identity remained elusive. Research by Zhou

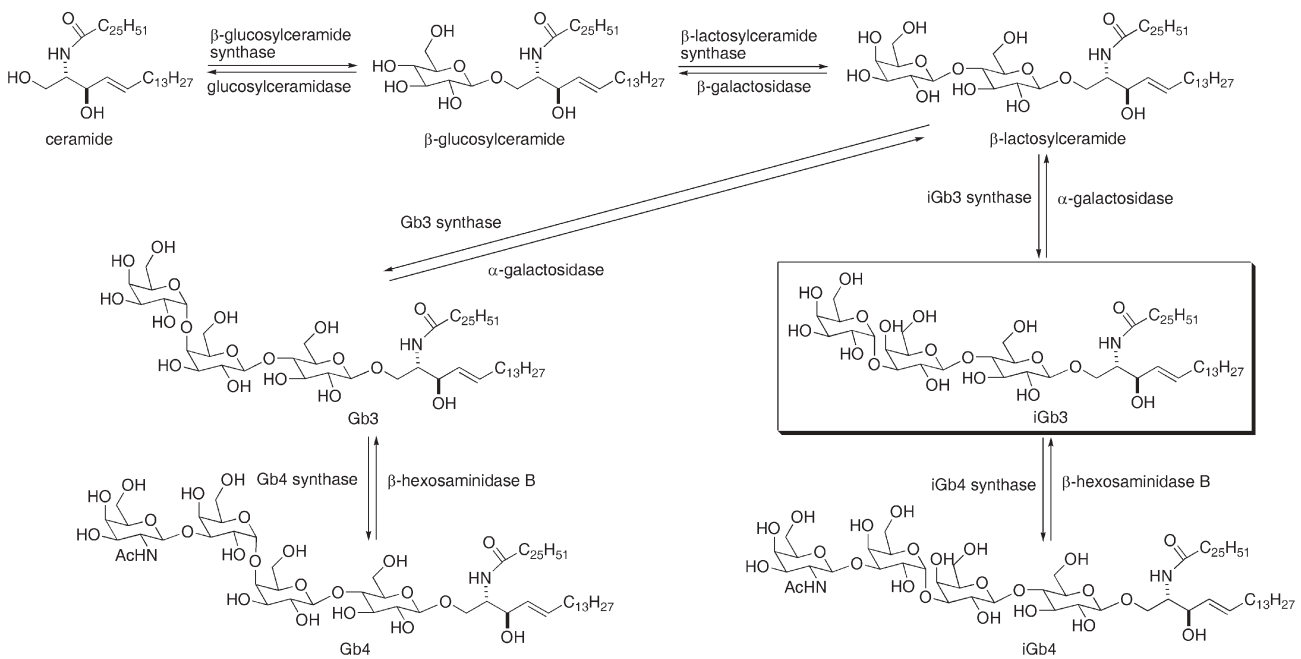

Fig. 11 Glycolipid processing pathways associated with Gb4 and iGb4. Enzymes involved in the synthesis or degradation of these glycolipids are described.

et al.[21] showed that mice deficient in β-hexosaminidase B were also deficient in NKT cells. The enzyme β-hexosaminidase B cleaves *N*-acetylgalactosamine groups from glycolipids and is involved in the degradation of globosides and isoglobosides; specifically, this enzyme converts Gb4 to Gb3 and iGb4 to iGb3 (Fig. 11). NKT cell deficiencies attributed to lack of β-glucosylceramide and β-hexosaminidase B narrowed considerably the pool of potential candidates for the natural antigen. β-monoglycosylceramides had been examined and showed no NKT stimulatory properties nor did β-lactosylceramide. Zhou, *et al.*[21] examined cytokine production of NKT cells stimulated by Gb3 and iGb3, and found that only iGb3 caused stimulation. For these studies iGb3 was obtained synthetically, enzymatically as well as isolated from cat intestines. This group also demonstrated that iGb4 only caused stimulation of NKT cells in the presence of β-hexosaminidase B. Notably, while glycolipids known to stimulate NKT cells prior to this study all contained an α-linkage between the proximal sugar and ceramide, iGb3 has a β-linkage at this position. Nevertheless, the distal sugar in iGb3 is an α-galactose and this may result in presentation of an epitope similar to that from glycolipids like KRN7000. An additional significant observation is that CD1d–TCR interactions distinguish between the different substitution patterns in Gb3 and iGb3, which highlights the high level of specificity of this interaction.

The studies of Zhou and *et al.*[21] demonstrate that iGb3 is necessary for positive selection of NKT cells in the thymus. However, the role of iGb3 and other potential endogenous antigens in regulating NKT cell responses is only partially understood. The innate immune system is finely tuned to recognize evidence of infection; in particular, lipopolysaccharide (LPS) from Gram-negative bacteria causes potent immune responses. These responses to LPS do not directly involve NKT cells. Nevertheless, through recognition of IL-12 and subsequent release of Th1 cytokines, NKT cells can potentiate immune responses to LPS.[36,37] For example, Brigl *et al.*[36] showed that blocking antigen presentation by CD1d renders NKT cells non-responsive to LPS suggesting that an endogenous antigen had to be presented to allow NKT cells to respond to LPS. In 2005, Mattner *et al.*[38] demonstrated that presentation of iGb3 by CD1d is required to allow NKT cells to indirectly respond to Gram-negative, LPS-positive bacteria through binding of IL-12 and release of Th1 cytokines. Because iGb3 plays a role in positive selection of NKT cells and allows NKT cells to participate in responses to infection, information about the regulation of iGb3 production and trafficking may allow an understanding of the underlying causes of defects in immune regulation.

3.2 Discovery of natural exogenous antigens for NKT cells.

Because most aspects of innate immunity were developed to detect and respond to microbial infection, it follows that NKT cells might play a role in surveying for bacteria. As noted above, it was discovered that NKT cells respond indirectly to LPS from Gram-negative bacteria. However, the question remained if NKT cells could recognize and respond directly to glycolipids from bacteria.

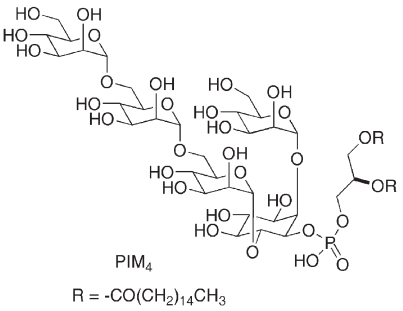

Fig. 12 Structure of PIM_4, a glycolipid from mycobacteria that stimulates NKT cells.

The first report of bacterial glycolipid mediated stimulation of NKT cells came from Schaible and coworkers in 2004.[39] Glycolipid presentation proteins related to CD1d have been shown to bind and present glycolipids from mycobacteria (*e.g. Mycobacterium tuberculosis* and *M. leprae*).[40] Schaible and coworkers purified glycolipids from mycobacteria and found that PIM_4 (Fig. 12) stimulated modest cytokine release from NKT cells as compared to stimulation with KRN7000. Interestingly, PIM_2 (lacking two of the mannose units of PIM_4) does not stimulate a response from NKT cells. This result argues that, similar with iGb3, the TCR on NKT cells can recognize sugars distal from the lipid portions of glycolipids sequestered by CD1d.

The outer membranes of most Gram-negative bacteria are comprised primarily of LPS. As described above, the innate immune system is well tuned to recognize LPS, and NKT cells play an indirect role in this response. However, there are numerous Gram-negative bacteria that do not produce LPS, and the structures of the lipids making up their outer membranes are only partially understood. The structures of glycolipids from one non-LPS-producing Gram-negative bacterial family, *Sphingomonadacaea*,[41,42] were elucidated primarily from the work of Zähringer and coworkers[43,44] with contributions from Kawasaki *et al.*[45] These glycolipids were termed glycosphingolipids 1, 3 and 4 (GSL-1, GSL-3, and GSL-4) and are shown in Fig. 13; the relative stereochemistry of the sphinganine portion of the molecule was determined as *erythro* but the absolute configuration was not established. Because in general sphingosines and sphinganines isolated from biological sources have D-*erythro* configurations (derived from L-serine) this is the stereochemistry given in Fig. 13. Glycosphingolipids from *Sphingomonadacaea* have also been isolated that contain galacturonic acid in place of glucoronic acid in GSL-1.[46]

A notable feature of the glycolipids from the *Sphingomonadacaea* family is the α-glycosyl–ceramide bond; it resembles the corresponding structure in KRN7000. The primary difference in structure between the GSLs and KRN7000 is that the former contain either glucoronic or galacturonic acids. These similarities were noted, and glycolipids isolated from this family of bacteria were tested by three different research groups[38,47,48] for the ability to stimulate NKT cells. As expected these glycolipids proved to be active. Specifically, GSL-1[38,47,49] and GSL-4[48] have demonstrated

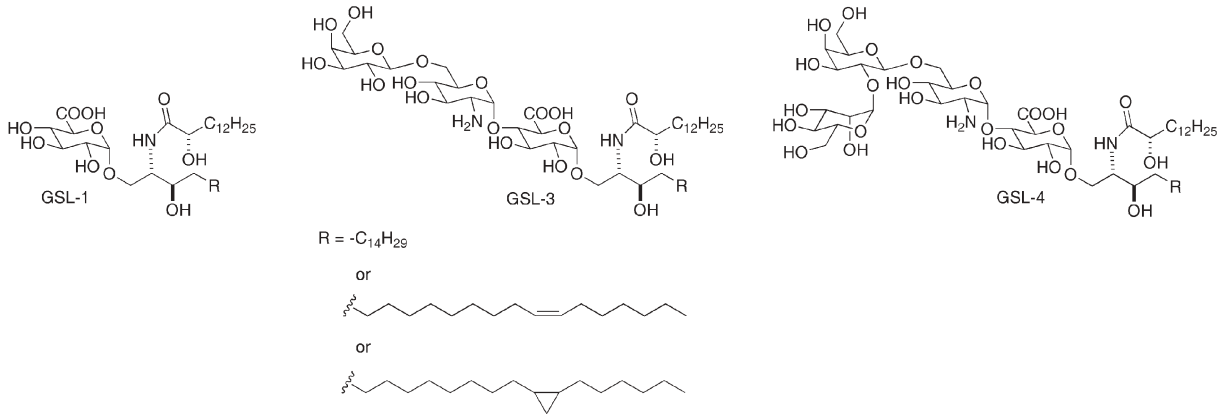

Fig. 13 Structures of glycolipids from bacteria in the *Sphingomonadacaea* family. GSL-1 and GSL-4 have been shown to stimulate NKT cells.

activity. To date, little structure–activity research has been reported with glycolipids from *Sphingomonadacaea*, and because KRN7000 and GSL-1 are similar, the same patterns derived from work with KRN7000 are expected. For example, β-glycuronosylceramides do not stimulate NKT cells.[38] The glycolipids isolated by Koezuka and coworkers[14,15] from *Agelas mauritianus* had hydroxyl groups at C2′ with the *R* configuration, while the C2′ configuration in GSLs from *Sphingomonadacaea* have an *S* configuration. From the structure–activity studies of the sponge-derived glycolipids reported by Morita *et al.*[10] in 1995 and more recent work with GSLs,[38,47] it has been demonstrated that this hydroxyl group plays an insignificant role in NKT cell stimulation.

Bacteria from the *Sphingomonadacaea* family are not widely held to be common human pathogens. Nevertheless, there are reports of pathogenicity,[50,51] and in the context of NKT cell studies, model infections have been established in mice.[38] However, it is improbable that the synthetic machinery necessary for formation of GSLs evolved only in the *Sphingomonadacaea* family. In fact, α-glycosylceramides have been isolated from other organisms including the bacteria *Arcocella aquatica* and *Flectobacillus major*[52] and the protist *Thraustochytrium globosum*.[53] Identification of glycosphingolipids in these bacteria, along with the fact that bacteria in *Sphingomonadacaea* are found in marine environments, suggests that the α-galactosylceramides initially isolated from a marine sponge may have bacterial origins.

In addition to bacteria in the *Sphingomonadacaea* family, there are multiple types of bacteria that are Gram-negative, non-LPS-producing bacteria. This characteristic is found in some important human pathogens, including bacteria from the genus *Ehrlichia*,[54] a bacterium related to the cause of typhus in humans. Mattner *et al.*[38] demonstrated that bacteria from this genus also stimulated NKT cells directly. It is possible that other non-LPS producing bacteria produce glycolipids that stimulate NKT cells and that persistent infection with these types of bacteria would result in biasing of immune system towards inflammatory Th1 responses.

Conclusions

NKT cells have been shown to play a major role in controlling immune responses, and the capacity of controlling NKT cell responses for treatment of human diseases will depend in part on an understanding of how glycolipids are presented by CD1d and recognized by NKT cells. From the initial isolation of glycolipids from a marine sponge by Koezuka and coworkers[14,15] to the identification of natural antigens, great strides have been made in this field. Nevertheless, many questions remain unanswered. For example: how is iGb3 synthesis and trafficking regulated? Are some human diseases related to defects in synthesis and trafficking of iGb3? Are there other endogenous antigens for NKT cells? Other than the bacterial glycolipids identified as antigens for NKT cells, are there other antigens produced by bacterial or parasitic pathogens? Can persistent bacterial infections and their effects on NKT cells cause mis-regulation of the immune responses? Can glycolipids be used medicinally to control immune functions in humans? Answers to these questions will involve research in immunology, microbiology and glycolipid chemistry. Research with NKT cell responses to glycolipids has progressed rapidly, from studies with marine natural products to identification of natural antigens. It is anticipated that progress will continue and new insights into glycolipid influences on the immune system will be realized.

References

1 M. Kronenberg and A. Rudensky, *Nature*, 2005, **435**, 598.
2 M. Kronenberg, *Annu. Rev. Immunol.*, 2005, **23**, 877.
3 L. Van Kaer, *Nat. Rev. Immunol.*, 2005, **5**, 31.
4 K. O. A. Yu and S. A. Porcelli, *Immunol. Lett.*, 2005, **100**, 42.
5 A. Jahng, I. Maricic, C. Aguilera, S. Cardell, R. C. Halder and V. Kumar, *J. Exp. Med.*, 2004, **199**, 947.
6 N. Okamoto, O. Kanie, Y.-Y. Huang, R. Fujii, H. Watanabe and M. Shimamura, *Chem. Biol.*, 2005, **12**, 677.
7 D. M. Zajonc, C. Cantu, III, J. Mattner, D. Zhou, P. B. Savage, A. Bendelac, A. Wilson, I and L. Teyton, *Nat. Immunol.*, 2005, **6**, 810.
8 M. Koch, B. S. Stronge, D. Shepherd, S. D. Gadola, B. Mathew, G. Ritter, A. R. Fersht, G. S. Bestra, R. R. Schmidt, E. Y. Jones and V. Cerundolo, *Nat. Immunol.*, 2005, **6**, 819.
9 D. M. Zajonc, I. Maricic, D. Wu, R. Halder, K. Roy, C.-H. Wong, V. Kumar and I. A. Wilson, *J. Exp. Med.*, 2005, **202**, 1517.
10 M. Morita, K. Motoki, K. Akimoto, T. Natori, T. Sakai, E. Sawa, K. Yamaji, Y. Koezuka, E. Kobayashi and H. Fukushima, *J. Med. Chem.*, 1995, **38**, 2176.
11 A. Uchimura, T. Shimizu, M. Morita, H. Ueno, K. Motoki, H. Fukushima, T. Natori and Y. Koezuka, *Bioorg. Med. Chem.*, 1997, **12**, 2245.

12 A. Uchimura, T. Shimizu, M. Nakajima, H. Ueno, K. Motoki, H. Fukushima, T. Natori and Y. Koezuka, *Bioorg. Med. Chem.*, 1997, **5**, 1447.
13 T. Natori, M. Morita, K. Akimoto and Y. Koezuka, *Tetrahedron*, 1994, **50**, 2771.
14 T. Natori, Y. Koezuka and H. Tatsuo, *Tetrahedron Lett.*, 1993, **34**, 5591.
15 K. Akimoto, T. Natori and M. Morita, *Tetrahedron Lett.*, 1993, **34**, 5593.
16 D. I. Godfrey and M. Kronenberg, *J. Clin. Invest.*, 2004, **114**, 1379.
17 E. Pál, T. Tabira, T. Kawano, M. Taniguchi, S. Miyake and T. Yamamura, *J. Immunol.*, 2001, **166**, 662.
18 T. Kawano, J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki and M. Taniguchi, *Science*, 1997, **278**, 1626.
19 S. Sidobre, K. J. L. Hammond, L. Bénazet-Sidobre, S. D. Maltsev, S. K. Richardson, R. M. Ndonye, A. R. Howell, T. Sakai, G. S. Besra, S. A. Porcelli and M. Kronenberg, *PNAS*, 2004, **101**, 12254.
20 T. I. Prigozy, O. Naidenko, P. Qazba, D. Elewaut, L. Brossay, A. Khurana, T. Natori, Y. Koezuka, A. Kulkarni and M. Kronenberg, *Science*, 2001, **291**, 664.
21 D. Zhou, J. Mattner, C. Cantu, III, N. Schrantz, N. Yin, Y. Gao, Y. Sagiv, K. Hudspeth, Y.-P. Wu, T. Yamashita, S. Tenenberg, D. Wang, R. L. Proia, S. B. Levery, P. B. Savage, L. Teyton and A. Bendelac, *Science*, 2004, **306**, 1786.
22 X. T. Zhou, C. Forestier, R. D. Goff, C. Li, L. Teyton, A. Bendelac and P. B. Savage, *Org. Lett.*, 2002, **4**, 1267.
23 R. M. Ndonye, D. P. Izmirian, M. F. Dunn, K. A. O. Yu, S. A. Porcelli, A. Khurana, M. Kronenberg, S. K. Richarson and A. R. Howell, *J. Org. Chem.*, 2005, **70**, 10260.
24 K. Miyamoto, S. Miyake and T. Yamamura, *Nature*, 2001, **413**, 531.
25 A. Chiba, S. Oki, K. Miyamoto, H. Hashimoto, T. Yamamura and S. Miyake, *Arthritis Rheum.*, 2004, **50**, 305.
26 M. Mizuno, M. Masumura, C. Tomi, A. Chiba, S. Oki, T. Yamamura and S. Miyake, *J. Autoimmun.*, 2004, **23**, 293.
27 R. D. Goff, Y. Gao, J. Mattner, D. Zhou, N. Yin, C. Cantu, L. Teyton, III, A. Bendelac and P. B. Savage, *J. Am. Chem. Soc.*, 2004, **126**, 13602.
28 S. Oki, A. Chiba, T. Yamamura and S. Miyake, *J. Clin. Invest.*, 2004, **113**, 1631.
29 J. S. Bezbradica, A. K. Stanic, N. Matsuki, H. Bour-Jordan, J. A. Bluestone, J. W. Thomas, D. Unutmaz, L. Van Kaer and S. Joyce, *J. Immunol.*, 2005, **174**, 4696.
30 K. O. A. Yu, J. S. Im, A. Molano, Y. Dutronc, P. A. Illarionov, C. Forestier, N. Fujiwara, I. Arias, S. Miyake, T. Yamamura, Y.-T. Chang, G. S. Besra and S. A. Porcelli, *PNAS*, 2005, **102**, 3383.
31 J. Schmieg, G. Yang, R. W. Franck and M. Tsuji, *J. Exp. Med.*, 2003, **198**, 1631.
32 G. Yang, J. Schmeig, M. Tsuji and R. W. Franck, *Angew. Chem., Int. Ed.*, 2004, **43**, 3818.
33 T. Toba, K. Murata, T. Yamamura, S. Miyake and H. Annoura, *Tetrahedron Lett.*, 2005, **46**, 5043.
34 J. K. Sandberg and H.-G. Ljunggren, *Trends Immunol.*, 2005, **26**, 374.
35 A. K. Stanic, A. D. De Silva, J.-J. Park, V. Sriram, S. Ichikawa, Y. Hirabyashi, K. Kayakawa, L. Van Kaer, R. B. Brutkiewicz and S. Joyce, *PNAS*, 2003, **100**, 1849.
36 M. Brigl, L. Bry, S. C. Kent, J. E. Gumperz and M. B. Brenner, *Nature Immunol.*, 2003, **4**, 1230.
37 Y. Kinjo and M. Kronenberg, *J. Clinical Immunol.*, 2005, **25**, 522–533.
38 J. Mattner, K. L. DeBord, R. D. Goff, C. Cantu, III, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, K. Hoebe, O. Schneewind, N. Ismail, D. Walker, B. Buetler, L. Teyton, P. B. Savage and A. Bendelac, *Nature*, 2005, **434**, 525.
39 K. Fischer, E. Scotet, M. Neimeyer, H. Koebernick, J. Zerrahn, S. Maillet, R. Hurwitz, M. Kursar, M. Bonneville, S. H. E. Kaufmann and U. E. Schaible, *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 10685.
40 M. Brigl and M. B. Brenner, *Annu. Rev. Immunol.*, 2004, **22**, 817.
41 Y. Kosako, E. Yabuuchi, T. Naka, N. Fujiwara and K. Kobayashi, *Microbiol. Immunol.*, 2000, **44**, 563.
42 K. Kawahara, H. Kuraishi and U. Zähringer, *J. Ind. Microbiol. Biotechnol.*, 1999, **23**, 408.
43 K. Kawahara, U. Seydel, M. Matsuura, H. Danbara, E. T. Rietschel and U. Zähringer, *FEBS Lett.*, 1991, **292**, 107.
44 K. Kawahara, H. Moll, Y. A. Knirel, U. Seydel and U. Zähringer, *Eur. J. Biochem.*, 2000, **267**, 1837.
45 S. Kawasaki, R. Moriguchi, K. Sekiya, T. Nakai, E. Ono, K. Kume and K. Kawahara, *J. Bacteriol.*, 1994, **176**, 284.
46 T. Naka, N. Fujiwara, E. Yabuuchi, M. Doe, K. Kobayashi, Y. Kato and I. Yano, *J. Bacteriol.*, 2000, **182**, 2660.
47 Y. Kinjo, D. Wu, G. Kim, G.-W. Xing, M. A. Poles, D. D. Ho, M. Tsuji, K. Kawahara, C.-H. Wong and M. Kronenberg, *Nature*, 2005, **434**, 520.
48 V. Sriram, W. Du, J. Gervay-Hague and R. R. Brutkiewicz, *Eur. J. Immunol.*, 2005, **35**, 1692.
49 D. Wu, G.-W. Xing, M. A. Poles, A. Horowitz, Y. Kinjo, B. Sullivan, V. Bodner-Narkevitch, O. Plettenburg, M. Kronenberg, M. Tsuji, D. D. Ho and C.-H. Wong, *Proc. Natl. Acad. Sci. USA*, 2005, **102**, 1351.
50 P.-R. Hsueh, L.-J. Teng, P.-C. Yang, Y.-C. Chen, H.-J. Pan, S.-W. Ho and K.-T. Luh, *Clin. Infect. Dis.*, 1998, **26**, 676.
51 C. Selmi, D. L. Balkwill, P. Invernizzi, A. A. Ansari, R. L. Coppel, M. Podda, P. S. Leung, T. P. Kenny, J. Van De Water, M. H. Nantz, M. J. Kurth and M. E. Gershwin, *Hepatology*, 2003, **38**, 1250–1257.
52 S. G. Bratrakov, V. I. Shienchenko and D. I. Nikitin, *Biochim. Biophys. Acta*, 1999, **1440**, 163.
53 K. M. Jenkins, P. R. Jensen and W. Fenical, *Tetrahedron Lett.*, 1999, **40**, 7637.
54 J. P. Olano and D. H. Walker, *Med. Clin. North Am.*, 2002, **86**, 375.