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Review

CD1d and natural T cells: how their properties jump-start the immune system

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Abstract. Cellular and humoral immune mechanisms recruited to defend the host from infectious agents depend upon the early immune events triggered by antigen. The cytokine milieu within which the immune response matures is the most important of many factors that govern the nature of the immune response. Natural T cells, whose function is controlled by CD1d molecules, are an early source of cytokines that can bestow type 1 or type 2 differentiative potential upon helper T lymphocytes. This review attempts to illuminate the glycolipid antigen presentation properties of CD1d, how CD1d controls the function of natural T cells and how CD1d and natural T cells interact to jump start the immune system. CD1d is postulated to function as a sensor, sensing alterations in cellular lipid content by virtue of its affinity for such ligands. The presentation of a neo-self glycolipid, presumably by infectious assault of antigen-presenting cells, activates natural T cells, which promptly release pro-inflammatory and anti-inflammatory cytokines and jump-start the immune system.

Key words. CD1d; glycolipid antigen; natural killer T cells; pro-inflammatory cytokines; anti-inflammatory cytokine; interleukin-4; interleukin-12; interferon-γ; granulocyte-macrophage colony-stimulating factor.

Introduction

Exquisite specificity for antigen, memory of them at subsequent encounters and tolerance to potentially provocative self are key features of the vertebrate immune system. T and B lymphocytes, the effector cells of the adaptive immune response, play a crucial role in imparting specificity, memory and self tolerance. An effective primary immune response to infectious agents, molecules or organisms, requires the concerted and regulated interplay of cells and molecules of the innate and adaptive immune systems. The cellular and humoral immune mechanisms recruited to defend the host from the infectious agent depend upon the early immune events triggered by antigen. Activation and elicitation of innate immune response is essential to limit the spread of the infectious agent in the host at early stages of infection. Through elicited humoral factors, activated effector cells of the innate immune system, communicate with the effectors of adaptive immunity [1]. Additionally, the innate effector cells sub-serve an essential function in presenting antigen to T lymphocytes [2]. Antigen recognition by naïve CD4+ helper T lymphocytes leads them to differentiate into type 1 or type 2 effector cells. The differentiation pathway followed by activated CD4+ helper T lymphocytes depends on the cytokine milieu in which they develop [3, 4]. Natural T (NK T) cells, whose function is controlled by CD1d molecules (see table 1), are an early source of cytokines that can bestow type 1 or type 2 differentiative potential upon CD4+ helper T lymphocytes [5]. In conjunction with cytokines elicited by antigen-activated effector cells of the innate immune system, NK T cell-derived cytokines can control adaptive immune responses towards an infectious agent. Thus, this review attempts to illuminate the structure and function of CD1d, the immunological role of NK T cells and how the two interact to jump-start the immune system.

Table 1. The nature of lymphocytes.

Lymphocyte subset	Phenotype	Antigen	Immune function
Th	CD4+8–, TCR$\alpha\beta^{high}$	MHC class II + peptide	regulates cellular versus humoral effector function
CTL	CD4–8+, TCR$\alpha\beta^{high}$	MHC class I + peptide	adaptive cell-mediated immunity
NK T	CD4+8–, CD4–8–, TCR$\alpha\beta^{int}$, $CD5^{high}$, $CD44^{high}$, CD122+, $CD161^{high}$, $Ly6C^{high}$	CD1d + glycolipid	immune regulation
NK	CD4–8–, TCR$\alpha\beta$–$CD44^{high}$, CD122+, $CD161^{high}$	MHC unrestricted	viral and tumour immunity

Overview of an Immune Response

Effector leukocytes of the innate immune system include phagocytes and natural killer (NK) cells. They recognise specific molecular patterns on the infectious agent. Examples of molecular patterns expressed by pathogens include lipoteichoic acids and lipopolysaccharides of Gram-positive and Gram-negative bacteria, respectively, glycolipids of mycobacteria, mannans of yeast, and double-stranded RNA of viruses. These molecular patterns are recognised by specific pattern recognition receptors expressed by the effector leukocytes. Some examples of pattern recognition receptors include lipopolysaccharide-binding proteins, CD14, Toll-like receptor, mannose-binding protein, surfactant protein-A and certain components of the complement cascade. Recognition of foreign patterns, patterns other than of self, leads to elicitation of extracellular and/or intracellular proteolytic cascades that eventually consume the infectious agent and hence limit its dissemination at an early stage of infection [6].

Additionally, foreign pattern recognition can activate the innate effector cell. A consequence of this activation process can be differentiation of the effector cell and/or the elaboration of humoral factors [2]. For example, internalisation of antigen by immature dendritic cells triggers their differentiation to professional antigen-presenting cells. During the process of differentiation they migrate from tissues, the homing site of immature dendritic cells, to secondary lymphoid tissues such as the draining lymph node and spleen. Here, mature dendritic cells are essential for T cell antigen processing and presentation [2]. Additionally, both immature and mature dendritic cells elicit key immunomodulatory cytokines. Of these, interleukin (IL)-12 is the most notable. It has a wide variety of functions: induced interferon (IFN)-γ elicitation and elaboration of cytotoxicity by NK cells as well as polarisation of activated CD4+ T lymphocytes towards type I immunity are key roles attributed to this cytokine [7].

Phagocytosis, pinocytosis and/or endocytosis of the infectious agent by macrophages and immature dendritic cells are essential early immune processes required for T cell antigen processing and presentation. Antigen processing and presentation are prerequisites for T cell antigen recognition, activation and effector function [2]. Internalisation of infectious agents brings antigen to endosomal/lysosomal vesicles where they meet antigen presenting molecules. The endosomal/lysosomal vesicles enriched in antigen presenting molecules are called class II-enriched vesicles and the major histocompatibility complex (MHC) class II compartment. The predominant antigen-presenting molecules in this compartment are the class II molecules encoded by the *MHC*, hence their name [8]. The MHC class II compartment also contains another class of antigen-presenting molecules collectively called CD1 [9–11]. Thus, the delivery of infectious agents to the MHC class II compartment followed by their processing provides a pool of derived products, some of which are presented to T cells by the antigen-presenting molecules.

MHC class II belong to the classical peptide antigen-presenting molecules. They present peptide antigens to CD4+ helper T (Th) lymphocytes. Naive CD4+ Th cells upon recognising antigen differentiate into Th1 or Th2 effector cells. The decision to follow the Th1 or Th2 developmental pathway depends on the cytokine milieu in which activation and differentiation of naive Th lymphocytes occur. Thus, in the presence of IL-12 and/or IFN-γ, activated Th cells differentiate into Th1 effectors, whereas in the presence of IL-4 they develop into Th2 cells (fig. 1). It is in this context that NK T cells are predicted to have a critical physiological role because they produce large amounts of cytokines, most notably IL-4, promptly, within 60–90 min of stimulation in vivo through their antigen-specific receptors [12]. Recent studies have suggested that NK T cells also play an important role in cross-talk between components of the innate immune system as well as the adaptive system (fig. 1) [13–15]. These cross-talks are facilitated by cell-cell interactions as well as by pro-inflammatory and anti-inflammatory cytokines secreted by NK T cells upon their activation by antigen.

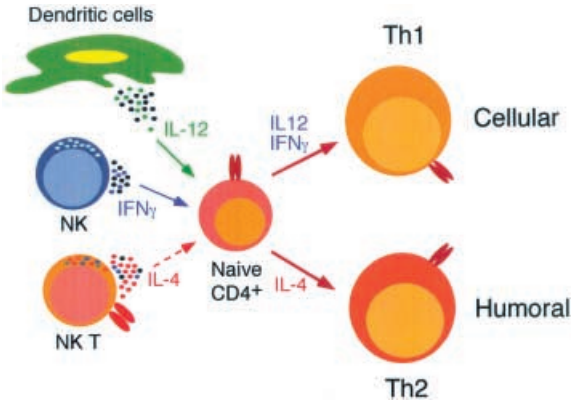

Figure 1. An overview of an immune response. Antigen recognition by naive CD4+ T lymphocytes leads them to differentiate into type 1 (Th1) or type 2 (Th2) effector cells. The differentiation pathway followed by activated CD4+ Th lymphocytes depends on the cytokine milieu in which they develop. Thus, in the presence of IL-12 secreted by dendritic cells and/or IFN-γ secreted by activated NK cells, activated Th cells differentiate into Th1 effectors. Likewise, in the presence of IL-4 whose source is thought to be NK T cells, activated Th cells develop into Th2 effectors. Solid arrows represent established source and function of the soluble factors, whereas the dashed arrow indicates potential source.

Thus this review focuses specifically on CD1d of both mice and humans and draws upon parallels as well as dissimilarities with other CD1 molecules where essential. Whereas diverse T cells react to CD1d, the focus here is on a subset of these that secrete IL-4 upon in vivo activation; in mice they express the invariant Vα14Jα15 T cell receptor (TCR) and in humans Vα24JαQ.

CD1d, a glycolipid-presenting molecule

A series of studies led to the identification of CD1d as the antigen presenting molecule that controls NK T cell development and function. In the first of these studies, NK T cells failed to develop in β_2-microglobulin (β_2m)-deficient mice. Adoptive transfer of wild-type foetal liver cells, as the donor for haematopoietic stem cells, into irradiated β_2m-deficient mice reconstituted the thymus with NK T cells [16–18]. Additionally, unlike the ontogeny of mainstream CD4+ and CD8+ T lymphocytes, the MHC type of the donor did not alter NK T cell development. These data suggested that the development of NK T cells is under the control of a conserved molecule(s) that associates with β_2m and is expressed by haematopoietic cells of the thymus [17]. Moreover, the ontogeny of NK T cells depended on the expression of the β_2m-associated molecule by CD4+8+ thymocytes [19]. Curiously, this is unlike the development of mainstream CD8+ and CD4+ cells, which require the expression of MHC class I and class II molecules by the thymic epithelium [20, 21].

Three groups of β_2m-associated molecules expressed by CD4+8+ thymocytes are known and include the non-classical MHC class I-like molecules, H2Qa2 and H2TL, as well as CD1d [22–26]. H2Qa2-negative and H2TL-negative thymocytes support NK T cell ontogeny and function [16, 27]. Thus, the ontogeny and function of NK T cells were predicted to be controlled by CD1d. NK T cell-derived hybridomas were specifically activated and hence stimulated to secrete IL-2 when co-cultured with cells infected with a recombinant vaccinia virus carrying the cDNA for mouse CD1d1 (rVV-CD1d1) but not rVV-H2K^b, a classical antigen-presenting molecule [22]. Similarly, freshly isolated thymic NK T cells co-cultured with cells infected with rVV-CD1d1 were activated to secrete IL-4 [22]. Finally, mice rendered CD1d deficient, through induced mutagenesis by homologous recombination, did not develop NK T cells [28–30]. These data conclusively demonstrated that CD1d indeed controls NK T cell ontogeny and function.

CD1d belongs to a group of evolutionarily conserved antigen-presenting molecules collectively called CD1 [31]. On the basis of nucleotide and amino acid sequence homology, CD1 is divided in two groups: group I CD1, consistsing of CD1a, CD1b, CD1c and CD1e, and group II, comprising CD1d [32]. Most mammals studied express CD1d [33–36]; it is yet to be found in miniature swine and guinea pigs [37, 38]. In humans, CD1d is one of four functional CD1 isotypes [33, 39, 40], whereas in mice [34] and rats [41], it is the only representative. Mice carry two CD1d genes, *CD1D1* and *CD1D2* [34]. *CD1D2* is a pseudogene [42]; hence, targeted disruption of *CD1D1* was sufficient to render mice CD1 deficient and hence defective in NK T cell development and function as well [30].

The expression of CD1d is tissue restricted. It is expressed by specialised antigen-presenting cells such as splenic dendritic cells and marginal-zone B lymphocytes [43]; marginal-zone as well as a novel subset of follicular B cells express higher levels of CD1d [44]. CD1d is additionally expressed by CD4+8+ double-positive thymocytes [19, 23, 45, 46], hepatocytes [47, 48] and *albeit* controversial [48], also by intestinal epithelium [45, 47, 49]. In all these cell types, CD1d expression is constitutive, and does not need inductive factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4, factors known to up-regulate the low levels of human group I CD1 expression on dendritic cells. Unlike group I CD1, CD1d expression is not up-regulated by GM-CSF and IL-4 [46]. Notwithstanding, the expression of mouse CD1d1 moderately increases in the presence of GM-CSF and IL-4 [48]. Whether other factors induce CD1d expression is currently unknown. In this regard, it is noteworthy that peritoneal macrophages elicited by thioglycolate express high levels of CD1d1 (A.D. De Silva and S. Joyce, unpublished data); whether the high levels of CD1d1 expression was due to factors

released by macrophages, other leukocytes or tissue cells surrounding the peritoneal cavity is currently not known. Strikingly, CD1d is not expressed by the cortical epithelium of the thymus, a site where MHC class I and class II expression is essential for positive and negative selection of mainstream T lymphocytes. Whereas circulating human monocytes as well as B and T lymphocytes express CD1d [46], these cell types in mice do not. The tissue-restricted expression of CD1d has implications for the ontogeny and function of NK T cells.

CD1d structure dictates its function

Phylogenetically, the primary structure of CD1d is distantly related to the *MHC*-encoded class I and class II antigen-presenting molecules [5, 50]. Akin to *MHC* class I genes, *CD1D* encodes the α chain of CD1d, which noncovalently associates with β_2m during assembly [34, 43]. The nascent α chain is ~33,000 Da [43] which is modified by four (in human) or five (in mouse) *N*-linked oligosaccharides [33, 34]. Thus, the mature α chain is ~50,000–55,000 Da in molecular weight [34, 43; A.D. De Silva and S. Joyce, unpublished data]. The α chain of CD1d, akin to MHC class I molecules, consists of three extracellular domains, α1, α2 and α3, anchored to the plasma membrane through a transmembrane domain which is followed by a short cytosolic tail.

The three-dimensional structure of CD1d

The solution of the three-dimensional structure by X-ray crystallography revealed that mouse CD1d1 topologically resembles the classical MHC class I and class II antigen-presenting molecules [51]. Consistent with its domain organisation and its association with β_2m, the three-dimensional structure of CD1d closely resembles that of MHC class I molecules. Thus the membrane-distal α1 and α2 domains fold to form a deep cleft. This cleft is bounded laterally by two α helices and at the floor by two β sheets, each made of four anti-parallel β strands; one α helix and one β sheet are contributed by each domain. The α3 domain and β_2m attain an immunoglobulin-like fold; they interact extensively with each other and also with the underside of the β sheet floor of the α1α2 superdomain. Because the primary structures of CD1d homologues and its paralogues (group I CD1) are highly conserved, the three-dimensional structures of all known CD1 molecules are predicted to closely resemble that of CD1d1 [31, 50].

The antigen-binding site of CD1d

The cleft formed by the design of the α1α2 superdomain is predicted to be the antigen-binding site of CD1d, based on the finding that the X-ray defraction data contained extra electron density within this cleft, which could not be solved from the known primary structures of the CD1d1 α chain and β_2m [51]. Careful examination of the extra electron density suggested the presence of an aliphatic hydrocarbon chain within the cleft. Extra electron density has also previously been observed in the crystal structures of human MHC class I molecules, HLA-A2 and HLA-A68 [52–54]; a human cell line expressing the MHC class I molecules was the source of HLA-A2 and HLA-A68 used in the X-ray crystallographic studies. Biochemical studies following the extraction of the low- molecular weight fraction associated with MHC class I molecules have revealed that the extra electron density observed in the crystal structures was the naturally processed peptide ligands that associate with these antigen-presenting molecules during their assembly [55, 56]. Moreover, point mutations that alter amino acid residues within the putative antigen binding site of CD1d1 affect the presentation of a glycolipid antigen to an NK T cell hybridoma [57]. Taken together, the data provide compelling evidence that the cleft of CD1d1, occupied by the extra electron density in its crystal structure, is most likely the antigen-binding site of CD1 molecules.

Topological similarities notwithstanding, the three-dimensional structure of CD1d1 differs from that of an MHC class I molecule; most of these differences are found in the cleft [51]. The antigen-binding site of CD1d1 is narrow at the opening that leads into two deep pockets lined exclusively by non-polar residues. MHC class I molecules, on the other hand, have wider access to their cleft that is lined indiscriminately by polar and non-polar amino acid residues [58]. The opening to the CD1d1 cleft has interesting structural features. It begins approximately at the centre of the antigen-binding site and extends to the carboxyl terminus of the cleft [51]. It is bounded at both ends, akin to the class I antigen-binding site, and thus is distinct from the open-ended groove of class II molecules. Additionally, pockets A – F observed in class I grooves that accommodate side chains of peptides have fused in CD1d1 to form two deep pockets, the larger A′ and the smaller F′ pockets. The A′ pocket is largely covered at the top by interactions between residues of the α1 and α2 helices, i.e. a roof is formed over pocket A′. Thus, the hydrophobic antigen-binding site of CD1d is accessible to the ligand through a narrow opening [51]. These differences in the physicochemical properties of the CD1d1 and class I antigen-binding sites have functional consequences. The former specialises in glycolipid and the latter in peptide antigen presentation to specific T cells.

The ligands of CD1d

The structural feature of CD1 molecules discussed above and their ability to control T cell responses discussed below suggest that they can function as antigen-presenting molecules. The best evidence for an antigen-presenting

function of CD1 comes from studies of human group I CD1 molecules. Human T cells reactive to mycobacterial (*Mycobacteria tuberculosis* and *M. leprae*) antigens can be established in vitro from peripheral blood leukocytes obtained from infected as well as uninfected individuals. Derivation of such T cells required repeated stimulation with antigen in the presence of GM-CSF- and IL-4-activated monocytes [59]. The resulting T cell specificity was shown to be controlled by CD1b and CD1c [59, 60]. The search for mycobacterial antigens presented by these group I CD1 molecules revealed that they are lipid and glycolipid components (table 2) of the pathogen cell wall. CD1b-restricted mycolic acid was the first identified lipid antigen; later, lipoarabinomannans, phosphatidylinositol (PI)-manans such as PI-mannose and PI-dimannoside, glucose monomycolate and, recently, dolicholphosphoryl mannose were identified as CD1-restricted antigens (table 2) [60–63]. Note that all these antigens have an amphipathic character comprised of a hydrophilic head group covalently linked to a hydrophobic fatty acyl or alkyl group.

Recently, another group of glycolipid antigens has been identified. It is presented to specific T cells by CD1b. These glycosphingolipids seem to function as autoantigens recognised by T cells derived from patients with multiple sclerosis, a chronic inflammatory autoimmune disease of the central nervous system. Using heterologous human dendritic cells pulsed with highly purified glycolipids, the antigen was identified as the glycosphingolipid GM1 [64]. Thus group I CD1 molecules can bind lipids whose hydrophobic aspect has diverse chemical features ranging from alkyl, poly-isoprenyl, fatty acyl and sphingoid groups (table 2).

The group II CD1d molecules have been reported to bind and present glycolipids and peptides to specific T cells. One approach to define the properties and function of the natural ligand(s) of an antigen-presenting molecule is to screen a random phage display library of peptides with the receptor. This approach, albeit powerful, introduces a bias in the property of the ligand, i.e. it assumes that the natural ligand(s) is peptidic in nature. Notwithstanding, useful information has emerged for the ligands of MHC class II molecules using phage peptide libraries. Likewise, a similar approach to define the natural ligand(s) of CD1d1 identificated a series of peptides that bind to the receptor with a reasonable association constant (K_d of $\sim 10^{-7}$ mol L^{-1}) [65]. Moreover, the identified peptides shared a hydrophobic binding motif. Interestingly, one peptide when presented by CD1d1 elicited a CD8+ cytotoxic T lymphocyte response in vivo. Peptides presented by CD1d, however, do not activate NK T cells [65], and hence are not discussed further here.

An α-anomeric form of glycosphingolipid, α-galactosylceramide (α-GalCer) possessing anti-cancer activity was discovered in a search for such drugs amongst natural products of the Orient [66]. α-Glycosphingolipid synthesis seems to be absent in all (including mammals), but a few organisms [66]. The anti-cancer activity of α-GalCer mimicked that of IL-12, and, in fact, required the participation of IL-12 [67, 68]. Studies directed towards delineating the mechanism of action of the α-glycosylceramides revealed that NK T cells are specifically activated by α-GalCer and α-glucosylceramide (α-GluCer) but not by α-mannosylceramide (α-ManCer) [69]. The in vivo function of the two active α-glycosylceramide requires the expression of CD1d1 by the antigen presenting cells as well as Vα14Jα15-positive mouse NK T cells [69, 70]. Moreover, several studies have demonstrated that human CD1d also binds and presents α-GalCer to Vα24JαQ-positive T lymphocytes [71–73], the human homologue of Vα14Jα15-positive mouse NK T cells. Thus one ligand presented by CD1d is a glycolipid containing a sphingoid base.

Additionally, a search for the natural antigen of NK T cells presented by CD1d1 identified a cellular phospholipid. This antigen was isolated from a total organic extract of cellular lipids. PI was identified as one NK T cell antigen using a commercial source of phospholipids to reconstitute biological activity in a panel of NK T cell hybridomas stimulated by a purified plate-bound CD1d1-immunoglobulin fusion protein [74]. Thus, akin to group I CD1, CD1d can also bind and present PI to T cells.

Another approach to elucidate the chemical nature of naturally processed ligand(s) presented by an antigen-pre-

Table 2. CD1 antigens

CD1	Antigen	Source	Reference
CD1a	undefined		
CD1b	mycolic acid, glucosemonomycolate, lipoarabinomannan, lipomannan, PI-dimannoside, PI-mannose	*Mycobacteria* spp.	57, 58
	GM1	self	59
CD1c	dolicholphosphorylmannose	*Mycobacteria* spp.; self	55
CD1d	α-glycosylceramide	synthetic; marine sponge	65–67

senting molecule entails the purification of the receptor in large quantities followed by the isolation and biochemical characterisation of the associated ligand(s) [75]. This approach assumes that the antigen-presenting molecule under study associates with a cellular ligand(s) during its assembly in vivo. The presence of an extra electron density within the antigen binding site of the first MHC molecule, HLA-A2, whose three-dimensional structure was solved by X-ray crystallography, suggested de novo association of class I molecules with cellular ligands [53, 55, 76]. Thus, the isolation of the associated ligands and their biochemical characterisation by Edman degradation and/or by mass spectrometry allowed the elucidation of the chemical properties of the naturally processed self peptides and antigens presented by numerous MHC molecules. It laid the first principles of peptide binding to MHC class I, class I-like and class II molecules [77–79].

The presence of the extra electron density in the crystal structure of CD1d1 (described above) suggested that it may associate with a cellular ligand [51]. Therefore, in one study [80], the above approach was applied, which entailed the purification of milligram quantities of CD1d1 in its native membrane-bound (mCD1d1) or soluble (sCD1d1) form expressed by mammalian cell lines. The CD1d1-bound ligand(s) was isolated by acid elution and fractionated by reversed-phase (RP)-chromatography. Characterisation of the predominant peak absorbing at 210 nm but not at 260 or 280 nm of the material acid eluted from mCD1d1 suggested that the natural ligand(s) was distinct from peptides or nucleic acid. That this peak did not contain peptides was confirmed by amino acid sequence analysis of a fraction of the low-molecular-weight material acid eluted from CD1d1. Thus Edman sequence analysis did not yield discernible information [S. Joyce, unpublished data]. Additionally, independent aminopeptidase and carboxypeptidase digestion of a fraction of the predominant peak eluting from the RP-chromatography followed by matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry-(MS) did not yield ions typically expected of loss of single or multiple amino acid(s) [A.S. Woods, S. Joyce and R.J. Cottor, unpublished data]. Thus, contrary to expectations from the ability of CD1d1 to bind peptides in vitro, the natural ligand(s) of CD1d1 may not be peptidic in nature.

The predominant peak eluting from the RP-chromatography described above as well as of peaks of low-molecular-weight material acid eluted from sCD1d1 were further analysed by MALDI-MS. Some of the resulting positive and negative ion spectra (see fig. 2) matched that of commercial PI isolated from bovine liver which contains stearic and arachidonic acids at C-atom 1 and 2 of glycerol [A.S. Woods, S. Joyce and R.J. Cottor, unpublished data]. This suggested that PI was part of the natural ligand associated with CD1d1 assembled in mammalian cells. Additional ions were observed in the spectra whose mass-to-charge ratio matched that of hexosamine, *N*-acetylhexosamine, multiple hexoses and phosphoethanolamine (see fig. 2), all components of glycosylphosphatidylinositol (GPI). Therefore, GPI was predicted to be the putative natural ligand of CD1d1.

Furthermore, sCD1d1 could be specifically radioactively labelled with precursors of GPI, [^{3}H]arachidonate, [^{3}H]ethanolamine, [^{3}H]-2-mannose [80] and [^{3}H]inositol [A.D. De Silva and S. Joyce, unpublished data], suggesting that the naturally processed ligand associated with CD1d1 was GPI. A limitation to such an analysis of lipid ligands is that unless the resulting radiolabelled ligand is isolated and characterised by biochemical means, the identity of the associated molecule cannot be ascertained with certainty. In other words, sCD1d1 labelling with [^{3}H]arachidonate and [^{3}H]ethanolamine, does not necessarily mean that they are incorporated in GPI. Thus [^{3}H]arachidonate and [^{3}H]ethanolamine could be incorporated into other phospholipids such as phosphatidylethanolamine, phosphatidylcholine and/or phosphatidylserine. Nevertheless, the specific incorporation of [^{3}H]-2-mannose and [^{3}H]inositol strongly suggests that CD1d1 is associated with GPI and possibly with PI as well.

Structure dictates function

Glycolipids are amphipathic in chemical character. The hydrophobic aspects of glycolipids made of fatty acyl, alkyl or sphingoid groups have different numbers of hydrocarbon chains, and hence differ in length. They can be branched or kinked due to unsaturation or epoxy or cyclopropyl modifications of the hydrocarbon chains. Thus, the deep hydrophobic cleft of CD1d is conducive to the binding of glycolipids through their hydrophobic hydrocarbon chain of diverse physico-chemical properties. The anchorage of glycolipids to CD1d through the lipid moiety allows the exposure of its hydrophilic aspect, viz., the carbonyl and carboxyl groups of fatty acid or the sugar component of glycolipids, to solvent, making it accessible for interaction with the antigen receptor (TCR) of NK T cells. Thus, studies of antigen presentation by group I and group II CD1 molecules have revealed the requirement for the hydrocarbon chains to bind CD1 and for specific sugars for recognition by the TCR [63, 81, 82].

Monosaccharides and oligosaccharides by themselves are not recognised by glycolipid-specific T cells. Therefore, CD1-restricted recognition of antigen entails the binding of the glycolipid antigen through its hydrocarbon chains, thereby presenting the sugar moiety to the receptor of specific T cells [63]. Moreover, detailed biochemical analysis designed to delineate the features of the hydrophobic core of the antigen required for binding CD1d1 revealed the following: (i) a single hydrocarbon chain, whether sphingoid or *N*-fatty acyl, was sufficient for binding CD1d1 [81]; (ii) the length of the hydrocarbon did not matter for binding CD1d1 and ranged from 8–24 car-

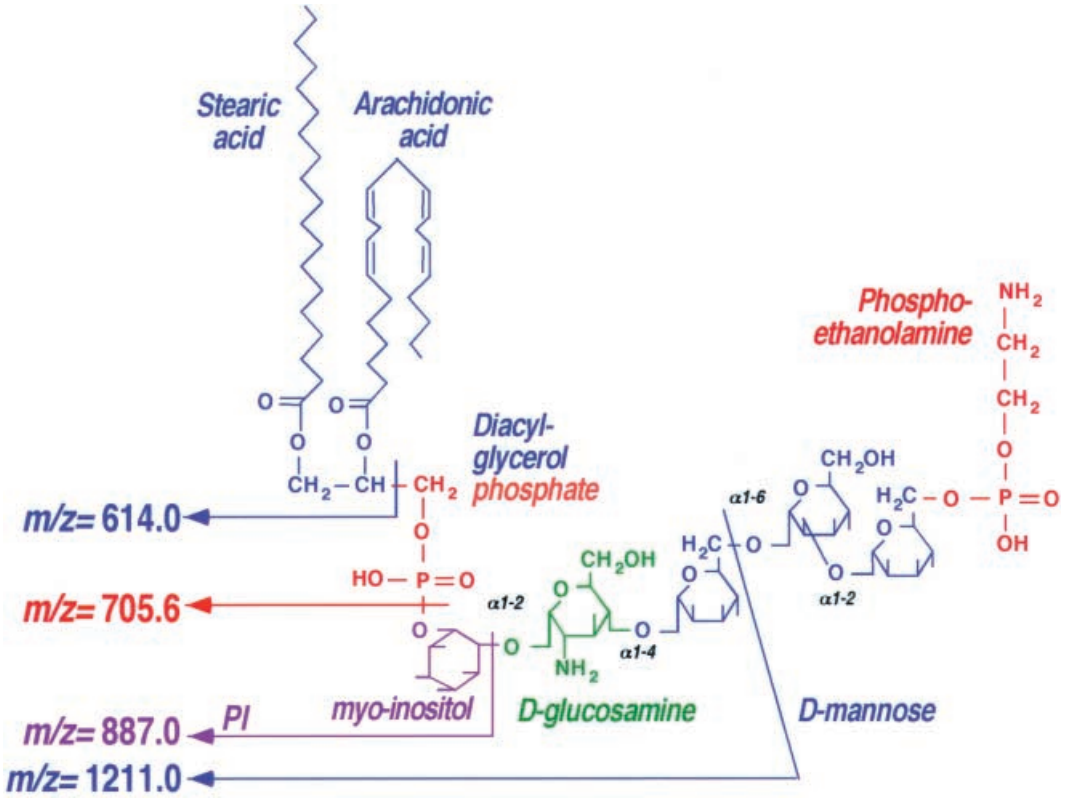

Figure 2. The structure of a mammalian glycosylphosphatidylinositol. The mass-to-charge ratio (*m/z*) of the positive and negative ions observed upon MALDI-MS analysis of the natural ligand isolated from membrane-bound and soluble mouse CD1d1 are indicated on the left. It offers one solution to the chemical nature of the CD1d1-associated ligand.

bon chains, the shortest and the longest tested [81]; (iii) kinked hydrocarbons (e.g. arachidonate) also bind CD1d1 [80]; (iv) the dissociation constant for the binding of glycosphingolipids and phospholipids to CD1d1 is similar, ~10^{-7} mol l^{-1} [80, 82]. Moreover, the affinity of the lipid for CD1d1 is similar to that of lipids for group I CD1 [80, 82, 83]. Additionally, the dissociation constant for lipid-CD1 interactions is similar to those reported for peptides and their cognate antigen-presenting molecules [84, 85]. Thus, the requirements for lipid-CD1 interactions are rather loose compared to peptide-MHC interactions; this is dictated by the physico-chemical features of the respective antigen-binding sites. In this regard, the resemblance of the CD1d1 antigen-binding site to that of non-specific fatty acid-binding protein is noteworthy [51].

T cells that recognise glycolipid antigen can discriminate between the different hexoses linked to the lipid [63, 69]. In nearly all cases, the first hexose forms the epitope for the specific T cell: glucose, galactose or mannose can form the epitopes [63, 69]. One study demonstrated that CD1d1-restricted NK T cells expressing Vα14Jα15/Vβ8.2 transgenic receptors recognised α-GluCer and α-GalCer but not α-ManCer [69]. Glucose and galactose differ from mannose by the axial (mannopyranose) and equitorial (glucopyranose and galactopyranose) orientation of the hydroxyl group of C-atom 2 of the pyranose ring. Additionally, glucose and galactose differ from each other by the axial (galactopyranose) and equitorial (glucopyranose) predisposition of the hydroxyl at C-atom 4. Thus hydroxyls at C-atom 2, 3 and 6 are common to both glucose and galactose and, hence, are predicted to impart specificity of the interaction between the presented glycolipid and the TCR. Because the above studies were performed with a monoclonal TCR, the possible presence of α-ManCer-reactive NK T cells remains possible. Similar discrimination of sugar epitopes as described for CD1d1-presented glycolipids are also observed in the interaction of glycolipids presented by group I CD1 to cognate TCR [63].

Topological biochemistry of antigen presentation by CD1d

It is evident from the studies described above that lipids form the natural antigen(s) of NK T cells. A search for the natural antigen of NK T cells has revealed a few features of lipid antigen presentation. The first of these is that the antigen is co-expressed by most cells that express CD1d; that is, CD1d expressed at the cell surface contains a bound cellular lipid that activates NK T cells and hybridomas and hence does not require exogenous addition of

antigen [10, 86–88]. Second, presentation of antigen to NK T cells does not depend on transporters-associated with antigen processing (TAPs) [87, 89] or H2Mα [S. Mendiratta, A. Boesteanu, S. Joyce and L. Van Kaer, unpublished data] molecules essential for antigen presentation by MHC class I [90] and class II [91], respectively. Both TAP1- and H2Mα-deficient mice develop NK T cells [92; Mendiratta et al. unpublished data]. Additionally, thymocytes from these animals as well as TAP1- and TAP2-deficient cell lines expressing CD1d1 are also able to activate Vα14Jα15-positive NK T cell hybridomas [89]. Thus, the rules for lipid antigen-presentation by CD1d are distinct from those of peptide antigen presentation by the classical antigen-presenting MHC class I and class II molecules.

Third, CD1 molecules, except CD1a and CD1e, contain at their carboxyl termini the Yxxϕ sequence [33, 34, 39, 40] that targets proteins to the low-pH endosome/lysosome compartment [93]. Mouse CD1d1 contains YQDI [34] and human CD1d contains YQGV [33] as the penultimate four amino acid residues. Both sequences target CD1d to the MHC class II-rich compartments [10, 11, 87, 94]. Additionally, the recognition of the presented antigen by a large majority of Vα14Jα15-positive NK T cells and hybridomas is affected by mutations that delete the Yxxϕ sequence or by those that alter it [10]. Thus, recycling of CD1d is essential for antigen presentation to most NK T cells. Nevertheless some Vα14Jα15-positive and most diverse TCR-positive CD1d1-reactive NK T cells see antigen assembled with CD1d in the anterograde secretory pathway [10], PI being one such cellular lipid antigen [74].

An appreciation of phospholipid and sphingolipid biosynthesis is essential to synthesise the available data on the cellular biochemistry of lipid antigen presentation into a model. These pathways are schematised in figs. 3 and 4 and briefly described in their respective legends. Of note with regard to CD1d assembly are the following.

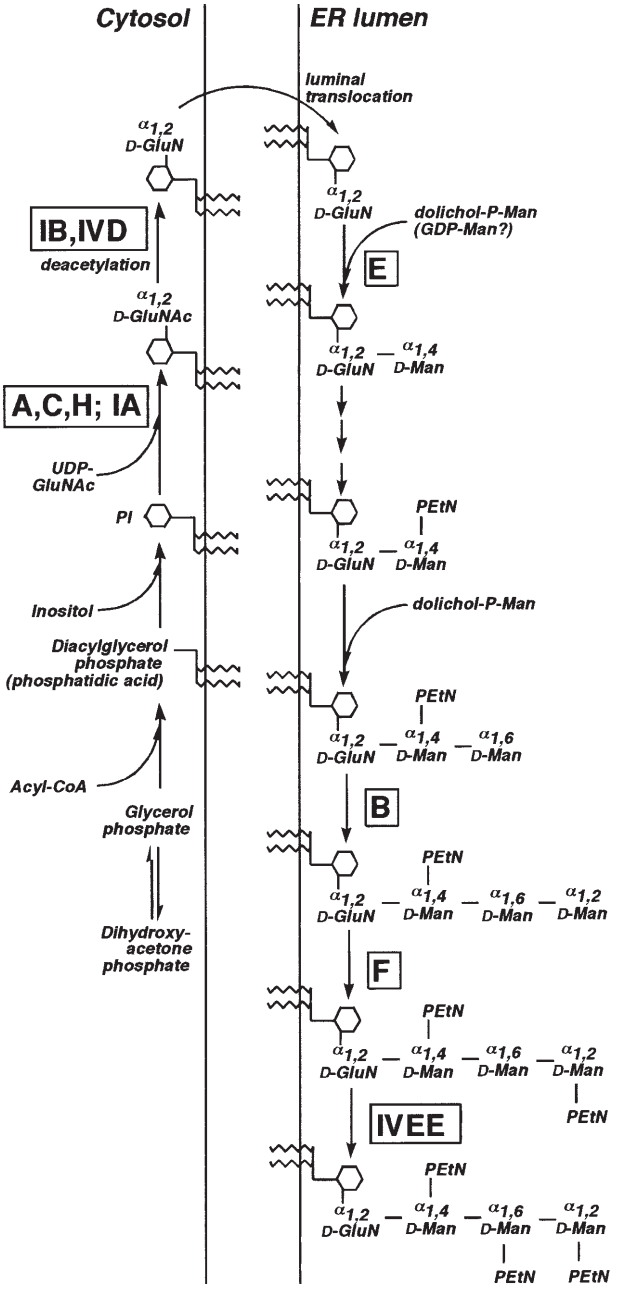

Figure 3. Biosynthesis of glycosylphosphatidylinositol. GPI serves to anchor proteins to the plasma membrane. A large body of information regarding the biosynthesis of GPI has been gathered from studies in trypanosomes, fungi and mammalian cells, from which the following scheme has emerged [246]. The biosynthesis of PI begins with the transfer of fatty acids from acyl-coenzyme A onto either dihydroxyacetonephosphate or glycerol-3-phosphate resulting in diacyl-glycerol-3-phosphate (phosphatidic acid) [247]. The addition of *myo*-inositol to diacyl-glycerol-3-phosphate-CDP results in PI. The step-wise glycosylation of PI and the addition of phosphoethanolamine (PEtN) to the third mannose (man_3) results in synthesis of the GPI core. PI is first glycosylated by uridine diphosphate (UDP)-*N*-acetyl-glucosamine (GluNAc) to form PI-α1,2-GluNAc [248], which is deacetylated to form PI-α1,2-GluN (GluN: glucosamine) [249]. Although not firmly established, prior or subsequent to acylation of the inositol ring of PI-α1,2-GluN, it translocates to the luninal leaflet of the endoplasmic reticulum (ER) where further glycosylation occurs by mannosylation [95, 250]. Thus, the GPI core consists of three linear mannoses of which the terminal residue is capped by the transfer of PEtN from phosphatidylethanolamine [251]. All terminal reactions in the biosynthesis of the GPI core occur in the ER lumen. Additionally, GPI free of linked proteins is present throughout the cell [96]. Murine mutant thymomas unable to synthesise GPI that belong to three complementation classes, A {PIG-A (PI-glycan-A) possibly a GluNAc transferase [252]}, C and H, suggest that the formation of PI-α1,2-GluNAc is catalysed by at least three enzymes [253]. Mutants of complementation class B are defective in the third GPI mannosyltransferase (PIG-B [95]) resulting in PI-glycans lacking the third mannose residue [254]. Class E mutants do not synthesize mannolipids and accumulate PI-α1,2-GluN due to a defect in dolicholphosphorylmannose synthase [255]. This mutant is also defective in *N*-linked glycosylation of proteins [255]. Group F mutants are defective in PIG-F, possibily a phosphoethanolamine-transferase [256], hence resulting in GPI lacking the terminal PEtN cap [253].

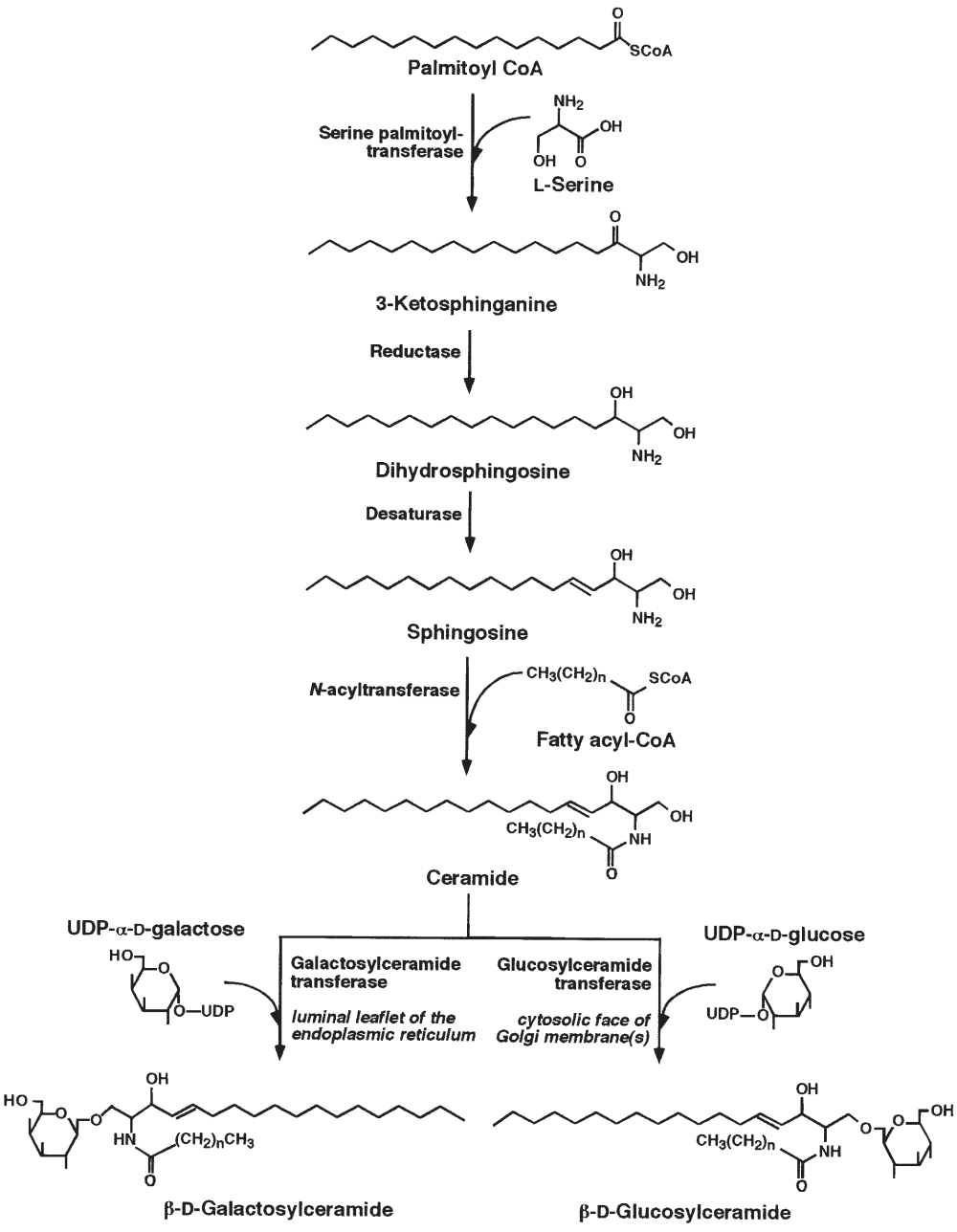

Figure 4. Biosynthesis of glycosphingolipids. Glycosphingolipid biosynthesis begins with the synthesis of ceramide on the cytosolic leaflet of the ER. The precursor for all cellular ceramide is dihydrosphingosine whose synthesis is initiated by the condensation of L-serine and palmitoyl-CoA. The condensation reaction is catalysed by L-serine palmitoyltransferase, an enzyme of the ER membrane. The resulting 3-ketosphinganine is reduced to dihydrosphingosine, a reaction catalysed by a reductase. Dihydrosphingosine is then rapidly *N*-acylated by an acyltransferase resulting in the formation of ceramide. All of these reactions occur on the cytosolic leaflet of the ER membrane. The glycosylation of ceramides results in the formation of glycosphingolipids [257, 258]. The major precursors for mammalian glycosphingolipids are β-D-glucosylceramide and β-D-galactosylceramide. β-D-glucosylceramide is synthesised by an enzyme UDP-α-D-glucose: ceramide-glucosyltransferase whose catalytic site is predisposed to the cytosolic side of the Golgi apparatus [99, 101]. In contrast, β-D-galactosylceramide is synthesised by an ER luminal enzyme, UDP-α-D-galactose: ceramide-galactosyltransferase [99, 100]. Note that both enzymes utilize the α-anomeric form of the UDP-charged sugars as the substrate to generate the β-anomeric form of the glycolipid.

(i) Although the synthesis of glucosaminylated PI occurs on the cytosolic leaflet of the endoplasmic reticulum (ER), mannosylation and phosphoethanolamine capping to generate the GPI core occur in the ER lumen [95]. Additionally, GPI free of linked proteins is present throughout the cell [96]. (ii) Dolicholphosphate-charged mannose, a donor for mannose for the biosynthesis of the GPI core as well as for protein glycosylation, and glucose, the donor for protein glycosylation, are present in the ER lumen [97, 98]. (iii) β-D-galactosylceramide is synthesised in the ER lumen [99, 100]. (iv) In contrast, β-D-glucosylceramide is synthesised on the cytosolic leaflet of the Golgi and flipped over to its luminal leaflet for the synthesis of complex glycolipids [99, 101]. Thus GPI and galactosylceramide are available for the assembly of CD1d in the ER and other membranous compartments of the cell. Additionally, dolicholphosphorylmannose and dolicholphosphorylglucose are available for the assembly of CD1d in the ER. Glucosylceramide, on the other hand, is available for CD1d assembly either in the Golgi apparatus or in an endocytic vesicle during recycling of CD1d from and to the plasma membrane.

A model for CD1d assembly in vivo

Being a type I integral membrane protein, akin to the classical antigen-presenting molecules for which much is known regarding assembly in vivo [8, 90], the assembly of CD1d begins as it is co-translationally inserted into the ER. Soon thereafter it binds calnexin, a step inferred from the association of the latter with a group I CD1 synthesised in the absence of β_2m [102] as well as with MHC class I molecules [103–105]. The role of calnexin in this association is probably to prevent aggregation of unassembled α chains. The monomeric α chain then assembles with β_2m forming a heterodimer that is receptive to ligands of the ER and/or the downstream vesicular compartment(s).

The ability to elute PI-glycans from both mCD1d1 and sCD1d1 [80] suggests that the association of the cellular ligand with CD1 occurred in the secretory pathway. Preliminary data indicate that association of the cellular ligand with CD1d1 might indeed occur in the ER or the early secretory pathway [A.D. De Silva, J.-J. Park, N. Matsuki, R.R. Brutkiewicz, M.E. Medoff, and S. Joyce, unpublished data]. The binding of GPI or a similar ligand to CD1d1 during its biosynthesis probably satisfies two structural demands, protection of the deep non-polar hy-

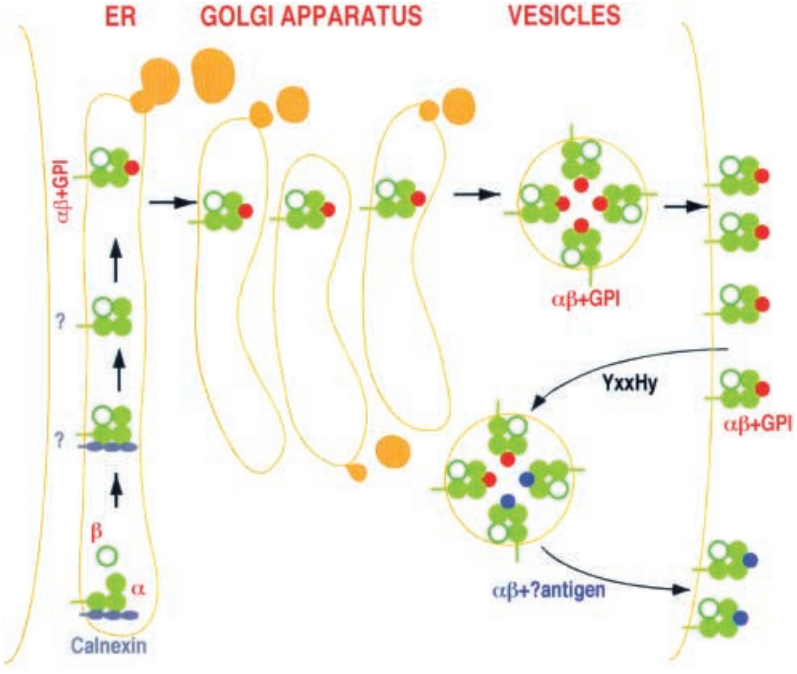

Figure 5. Predicted pathway for the assembly and traffic of CD1d molecules. Being a type I integral membrane glycoprotein, the folding and assembly of CD1d occur in the rough ER. Here, the folding of the CD1d α chain is assisted by calnexin. β_2m presumably associates with the folded α chain-calnexin complex. The α chain+ β_2m-calnexin complex forms a structure receptive to resident lipids and/or glycolipids. In the presented model, the glycolipid ligand is GPI because it was the major natural ligand isolated from membrane-bound and soluble mouse CD1d. Upon stable association, the CD1d (α chain and β_2m)-glycolipid complexes dissociate from calnexin, egress from the ER and negotiate the secretory pathway to the plasma membrane. Because of the internalisation sequence within the cytosolic tail of CD1d, it is rapidly internalised and recycled back to the plasma membrane. During its time in the endosome/lysosomal compartment, CD1d exchanges the lipid it bound in the ER for another, presumably cellular, glycolipid that is presented to Vα14Jα15-positive NK T cells. How CD1 molecules load glycolipids into their antigen-binding site in the ER during assembly and during their transit through the recycling compartments remains to be elucidated.

drophobic cleft from collapse as well as occupying it with a ligand that might be easily exchanged for an antigen in a late secretory vesicle. Thus GPI might play a chaperone-like role (fig. 5) akin to the function of MHC class II-associated invariant chain in class II assembly and traffic in vivo [8].

CD1d1 expression in PIG-A (refer to fig. 3 for a description of PIG mutants) mutants is normal. Additionally, Vα14Jα15-positive and – negative NK T cells recognise CD1d1 expressed by the mutant as effectively as they recognise wild-type CD1d1. Thus GPI, *albeit* a major natural ligand of CD1d1, is neither essential for the assembly of CD1d in vivo nor is it the natural antigen of NK T cells [88]. Therefore, in the absence of GPI, PI or another lipid (e.g. phosphatidylethanolamine, phosphatidylserine, dolicholphosphoryl-monosaccharides and β-D-galactosylceramide) present in the ER may substitute for the function of GPI in the assembly of CD1d in vivo. Following assembly of CD1d in the ER, it negotiates the secretory pathway and arrives at the MHC class II-enriched vesicles either directly from the trans-Golgi or via the plasma membrane. Targeting to the class II-enriched vesicles depends on the Yxxϕ internalisation sequence found at the cytosolic tail of CD1d [10, 11]. Here, CD1d meets the naturally processed antigen(s) and brings them to the plasma membrane for presentation to NK T cells (fig. 5) [10].

How lipid loading onto CD1d in the ER is accomplished and how these lipids are exchanged for antigen in endocytic vesicles remains an important unsolved question. Because lipids are membrane embedded, they need to be 'plucked out' of the membrane and loaded into the antigen-binding site of CD1d. This event might require an elaborate molecular machinery similar to the peptide-loading complex essential for peptide-antigen assembly with MHC class I and class II molecules [8, 90]. Alternatively, CD1d itself might play a role in loading itself with glycolipids. CD1d molecules have four conserved *N*-linked glycosylation sites, asparagines 20, 42, 109 and 166 [33, 34, 50]. All of these sites are predicted to be solvent exposed based on the three-dimensional structure of CD1d1 [51]. Preliminary data suggest that all five glycosylation sites in CD1d1, including the four conserved ones, are indeed modified [A.D. De Silva and S. Joyce, unpublished data]. One or more of the conserved glycosyl groups may function as a lectin(s), thereby binding to membrane-associated glycolipids, 'plucking' them out of the membrane and facilitating the loading of CD1d molecules; the loading could occur in *cis*, i.e. loading itself, or in *trans*, i.e. loading another CD1d molecule. Whatever the mechanism, its solution should provide insights into the basis for lipid-protein interactions in vivo and into the evolution of the lipid antigen-presenting system in vertebrates.

NK T cells

NK T cells are an unusual subset of lymphocytes. Phenotypically they express molecules on their cell surface characteristic of NK and of T cells, and hence their name. NK cell-specific markers expressed by NK T cells include CD161 (NKR-P1C, also known as NK1.1 of mice or NKR-P1A in humans) [87, 106, 107], some members of the Ly49 (a few express Ly49A or Ly49G2 and a large majority express Ly49C/I) family of proteins during development [108] and low levels CD16, which is up-regulated upon activation [109]. Of the T cell-specific markers, NK T cells express the TCR; most NK T cells express the $\alpha\beta$ TCR and are described in detail below, but some express the $\gamma\delta$ TCR [110, 111] whose biology remains unknown and hence is not discussed further. Additionally, NK T cells express markers shared by NK cells and activated T lymphocytes (table 1); these include CD5, CD44, CD69, CD122 (IL-2 receptor β chain) [106, 109, 112], Ly6C [113–116] and IL-7 receptor α (IL-7Rα) chain [112]. Moreover, akin to activated T lymphocytes, NK T cells express low levels of CD24 (heat stable antigen) [92, 117, 118] and CD62L (LECAM/MEL-14) [110, 115]. NK T cells also express ICAM-1 and LFA1-1 [119, 120]. Expression of these phenotypic markers is taken to indicate that NK T cells are in a state of chronic activation.

A proportion of NK T cells express CD4 co-receptors normally displayed by Th cells and the rest express neither CD4 nor CD8 [16, 106, 107, 117,121–124]. The functional consequence if any, of such a phenotypic dichotomy is presently unclear. Curiously, however, CD4 expression by NK T cells does not require functional MHC class II molecules during development [16]. Moreover, CD4 expression itself is not required for NK T cell ontogeny [16], suggesting that this co-receptor may be dispensable and non-essential for NK T cell biology.

NK T cell ontogeny

Thymic development of T lymphocytes occurs in waves; the first wave is of $\gamma\delta$ T cells followed by mainstream $\alpha\beta$ T cells [125]. Very few NK T cells are present at the birth of mouse pups; their development begins during postnatal lymphopoiesis and peaks around 6 weeks of age [16, 113]. NK T cells are thought to develop in the thymus and home to preferential secondary lymphoid organs [16, 122, 126, 127]. They account for about a million cells in each lymphoid organ except for their rarity in peripheral blood and the lymph nodes [16, 18, 123] or complete absence within the intraepithelial lymphocyte population [18]. They constitute 10–20% of mature T cells in the thymus, 20–30% of hepatic and bone marrow T cells, 0.5–1.0% of splenocytes, and 0.1–0.5% of lymph node and peripheral blood mononuclear cells [18, 128]. Addi-

tionally, NK T cells seem to be recruited to the peritoneum following intraperitoneal delivery of pathogens such as *Salmonella choleraesuis* and *Listeria monocytogeneses* [129, 130]. Thus NK T cells home to critical anatomical sites where they could execute their immunological function promptly.

NK T cells that develop in the thymus (NT^{thy}) are characterised by the expression of a highly restricted TCR repertoire [16, 27, 87, 106, 107, 113, 117, 121, 122, 124, 131–136] and the absence of CD8 co-receptor [16, 128]. The NT^{thy} TCR repertoire consists predominantly of the Vα14Jα15 (formerly Jα281 [137]; 80–90%) TCR α chain that preferentially pairs with Vβ8.2 (40–60%), Vβ7 (10–15%) or Vβ2 (4–6%) TCR β chain [16, 133]. Their development requires the expression of the β_2m-dependent CD1d1 molecule [16–18, 28–30] by haematopoietic cells of the thymus, the CD4+8+ double-positive thymocytes [19, 138]. Humans express the Vα24JaQ TCR α chain that predominantly pairs with the Vβ11 TCR β chain; both these TCR chains are homologous to the mouse Vα14Jα15 and Vβ8.2 receptors [87, 132–136]. As in mice, diverse TCR β chains pair with the Vα24JαQ TCR α chain of human NK T cells [132, 135, 136].

Interestingly, *nu/nu* mice deficient in a functional thymus gland develop NK T cells, but akin to mainstream T lymphocytes of *nu/nu*, they are present at very low numbers [107, 128, 139, 140]. NK T cells of *nu/nu* mice are present in the liver, spleen and bone marrow, constituting ~0.5% of hepatic mononuclear cells, ~0.5% of B cell-depleted splenic and ~0.2% B cell-depleted bone marrow cells [128]. NK T cells, NK1.1+CD3+, develop in *nu/nu* mice reconstituted with syngeneic bone marrow-derived cells. Additionally, syngeneic bone marrow transfer into an adult thymectomised and irradiated host results in the development of NK T cells [139, 141]. Curiously, they express CD8 co-receptors and are found in the liver and spleen of the reconstituted recipients [141]. Thus, in some quarters, these results have been taken to indicate extrathymic development of NK T cells. Extrathymic T lymphopoiesis does occur in normal mice; a large majority of the resulting T cells predominantly express CD8$\alpha\alpha$ co-receptor as opposed to the CD8$\alpha\beta$ and CD4 phenotype of thymic émigrés [142].

Recently, a second type of NK T (NT^{peri}) cells have been described. They develop independent of CD1d expression and most express CD8α co-receptor while the remainder are devoid of either CD4 or CD8 molecules [128]. Additionally, NT^{peri} differs from NT^{thy} in two key ways. (i) The former expresses a wider TCR repertoire [128] in contrast to the restricted TCR repertoire of NT^{thy} (described above). (ii) NT^{peri} cells are predominantly present within the spleen and bone marrow and are thought to originate from the bone marrow [128]. In this regard it is noteworthy that the CD8+ NK T cells are negatively selected in the thymus and hence are not present amongst thymocytes [16] or hepatic mononuclear cells. These findings can then explain why *nu/nu* mice develop NK T cells; they actually belong to the NT^{peri} lineage and hence may have an extrathymic origin. NT^{peri} cells are not discussed any further.

Preliminary results using genetically altered mice support a thymic origin for the IL-4-secreting NK T cells. Mice deficient in Janus kinase-3 (Jak3), the common cytokine receptor γ chain (γc)-associated kinase, lack mature NK T cells in the thymus, liver and spleen. Thymocyte-specific expression of Jak3 by the proximal promotor of Lck through transgenesis in Jak3-null mice rescues the NK T cell deficiency. Splenic NK T cells of thymocyte-specific Jak3 transgenic mice promptly secrete IL-4 when stimulated in vivo. Thus, akin to mainstream T cells, NK T cell development occurs in the thymus; thence the functionally mature NK T cells home to the peripheral lymphoid organs [M.E. Embers and S. Joyce, unpublished data]. That they home to the peripheral lymphoid organs is supported by the dramatically reduced numbers of hepatic NK T cells in LFA-1-deficient mice despite an almost normal contingent of thymic NK T cells [119, 120].

The thymocentric bias in NK T cell development notwithstanding, there is compelling evidence for extrathymic NK T cell ontogeny. One study documented rearrangement of *Vα14* and *Jα15* gene segments within day 9 mouse foetal liver progenitors. Temporally, this rearrangement precedes that of *Tcr* gene segments within mouse foetal thymus; here *Tcr* gene rearrangements occur around day 13 or 14 of gestation. Hence development is thought to occur in situ without the need for the thymus [143]. Additional independent data support an extrathymic NK T cell ontogeny [144]. Cytokine-supplemented cultures of wild type and *nu/nu* foetal liver develop NK T cells that express the invariant TCR α chain. Moreover, foetal liver cultures derived from β_2m-deficient mice (which lack MHC class I and other related molecules) or from wild type mice in the presence of CD1d-specific monoclonal antibody did not support the development of NK T cells. Additional data revealed that SCID (recombination-deficient but CD1d-positive) foetal liver reconstitutes NK1+ T cell development within β_2m-deficient (recombination-competent but CD1d-deficient) foetal liver cultures. Therefore, foetal liver progenitors have the potential to beget NK1+ T cells in situ, lending support for extrathymic NK T cell ontogeny [144].

Signals for NK T lymphocyte development

Their unique phenotypic features (see table 1 and their function, prompt secretion of immunoregulatory cytokines upon activation (table 3), distinguish NK T cells from mainstream T lymphocytes. Amongst naive lymphocytes, NK T cells represent a single antigen-reactive T

Table 3. Properties of cytokines/chemokines elicited by natural T cells

Cytokine/ chemokine	Source: natural T cell		Target cell(s)	Function(s)
	non-diabetic [12, 86, 117, 174–177]	diabetic [177]		
IL-2	+		lymphocytes	autocrine growth factor
IL-4	+++++	–	leukocytes	anti-inflammatory; Th2 and B cell activation and differentiation; immunoglobulin isotype switching to IgE; eosinophilia
IL-5	++	–	eosinophils	eosinophila
IL-10	++		leukocytes	anti-inflammatory; potent inhibitor of macrophage function
IL-13	+++++	–	leukocytes	anti-inflammatory; Th2 and B cell activation and differentiation; immunoglobulin isotype switching to IgE
IFN-γ	+++	+	leukocytes	pro-inflammatory; host defence against tumours and viruses; macrophage, NK and cytotoxic T lymphocyte activation
GM-CSF	+++++	+	myeloid cells	haematopoiesis; activation and differentiation of myeloid cells
TNF-α	++	–	leukocytes, endothelial cells	pro-inflammatory; local inflammation, activation of endothelial cells
TNF-β	++	–	leukocytes, endothelial cells	pro-inflammatory; cytotoxic; endothelial cell activation
MIP-1α	+++++	+	leukocytes	chemoattractant; activation
MIP-1α	+++	+	leukocytes	chemoattractant; activation
Lymphotactin	+++	–	lymphocytes	chemoattractant

lymphocyte population present at high frequency [145, 146] compared to that of any antigen-reactive T lymphocytes [147–149]. The phenotype ($CD44^{high}$ $CD62L^{null}$) of NK T cells suggests that they were recently activated. They home within the spleen around the CD1d+ marginal-zone B cells as well as within the liver where resident lymphocytes meet antigen. Additionally, the phenotype and frequency of NK T cells of mice maintained in normal and germ-free conditions remain unaltered [150]. Therefore, the activated phenotype and high frequency of NK T cells as well as their prompt response to antigen (discussed below) seem not to be due to constant confrontation with antigen in vivo. Thus NK T cells might be developmentally wired and maintained in vivo at or just below the threshold of activation so that they can promptly respond to antigen.

Their unique phenotypic and functional features have prompted studies on the signals that commit precursor thymocytes to the NK T cell lineage and the ontogeny of these T lymphocytes. Whereas much information is available on the development of mainstream T cells, very little is known regarding NK T cell ontogeny. A detailed fate map of mainstream T cells in the thymus based on the current knowledge is summarised and represented schematically in the upper panel of figure 6. In this scheme, there are three critical check-points: the first is the decision toward T lineage commitment; it follows the first wave of precursor expansion in the thymus. Commitment occurs at the CD25+ thymocyte stage or shortly thereafter. γc, the IL-7Rα chain and Jak3 provide critical signals for precursor expansion at the CD44+CD25+ stage in T lymphopoiesis. β selection controls the second check-point in T cell ontogeny. Signals critical for attaining this stage of T lymphopoiesis include those relayed by the TCRβ-pre-Tα heterodimer through Lck, Zap70, Syk, Sox4, Mek and Tcf1. The final checkpoint entails positive and negative selection of the MHC-restricted but self-tolerant TCR repertoire. Signals transmitted through the TCR and CD4 or CD8 co-receptors are critical at this stage of T lymphopoiesis. Such signals are transduced intracellularly by Lck, Zap70, Vav, Itk, Raf, Ras and Mek and involve two transcription factors, NFκB [A. Mora and M. Boothby, personal communication] and Irf1. Thence the functionally mature MHC-restricted self-tolerant T lymphocytes home to the peripheral lymphoid organs [reviewed in refs 151, 152–155].

Several of the signals required for the development of mainstream T cells are also required for NK T cell ontogeny. These include signals relayed through γc that are transduced intracellularly by Jak3. γc- and Jak3-deficient mice completely lack mature NK T cells [112, 118]; M. E. Embers and S. Joyce, unpublished data]. Neverthelesss transcripts of functionally rearranged Vα14Jα15 TCR α chain are detected amongst thymocytes of γc-deficient mice; these transcripts are absent amongst splenocytes and hepatic mononuclear cells [112]. Additionally, they

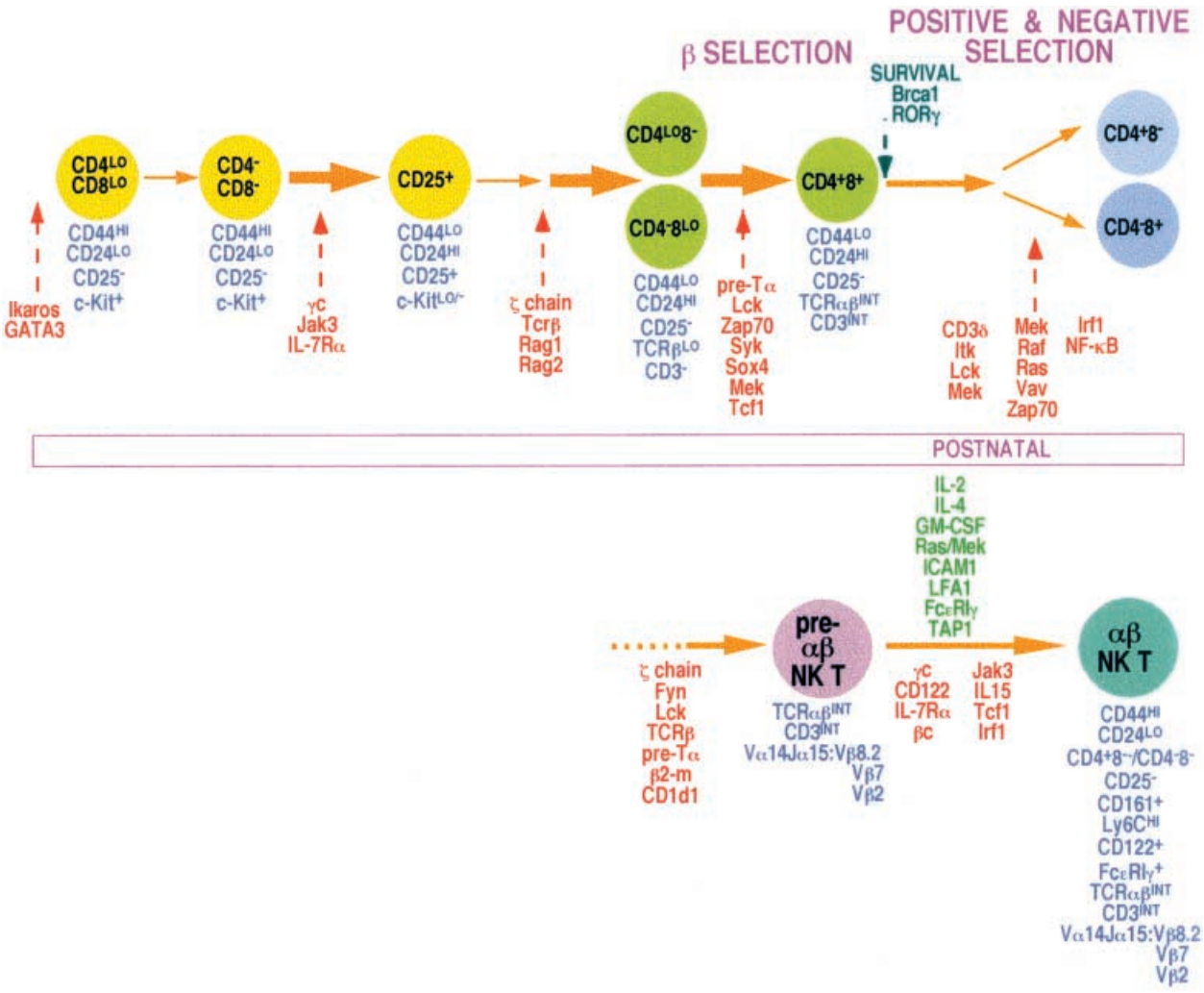

Figure 6. Major stages in mainstream and NK T cell ontogeny in the thymus. The developmental pathway of mainstream T cells is based on Rothenberg [151], Shortman and Wu [153], Kisielow and von Boehmer [152], Sebzda et al. [155], Kuo and Leiden [154], and references therein. Specific markers indicated below the cells distinguish each thymocyte lineage. Vertical dashed arrows indicate the mutation that affects onward development. The thickness of the horizontal arrows denotes waves of thymocyte expansion [151]. The factors that do not affect NK T cell development are indicated above the horizontal arrow. The precise progenitor that gives rise to NK T cells is currently unknown. The effects of null mutations in NK T cell development are based on published (see text) and unpublished studies [NFκB: A. Mora and M. Boothby; Jak3: M.E. Embers and S. Joyce; GM-CSF: N. Matsuki and S. Joyce].

also express the Vβ8 TCR β chain on the cell surface of CD4–8– double-negative thymocytes but lack expression of CD44, CD122, CD161 and IL-7Rα, markers characteristic of NK T cells [112]. These data further attest to the thymic development of NK T cells and identify a pre-NK T cell (fig. 6, bottom panel) [112].

The common cytokine receptor γ chain is utilised by IL-2, IL-4, IL-7, IL-9 and IL-15 as a component of their respective receptor complex [156]. Additionally, IL2Rβ (CD122) forms a part not only of the IL-2 receptor but also of the IL-15 receptor [157]; thus both IL-2 and IL-15 share IL-2Rβ and γc whereas cytokine specificity is imparted by their respective receptor α chains. Because IL-2Rβ-deficient mice lack NK T cells [158], and since an IL-2 deficiency did not affect NK T lymphopoiesis [118], IL-15-mediated signalling was thought to play a key role in NK T cell development. Thus both IL-15- and IL-15Rα-deficient mice have very low numbers of NK T cells in the thymus and the peripheral lymphoid organs [159, 160]. The requirement for IL-15-mediated signals for NK T cell growth and function is also shared by NK cells. In contrast to the latter, which is completely absent in the IL-15-deficient mice, NK T cells are reduced in number but not completely absent [159]. This finding might suggest that IL-15 plays a role in maintaining NK T cell homeostasis either by providing signals for cell survival or for cell growth and proliferation.

IL-2-, IL-4- and IL-7-mediated signals are not required for NK T cell development. But curiously, IL-7Rα deficiency recapitulates the phenotype observed in IL-15-

null mice with regard to NK T cell numbers, i.e. both strains of mice have dramatically lowered numbers of NK T cells [118, 159]. Signals relayed by IL-7Rα promote the first wave of thymocyte expansion and survival [161, 162]; this function of IL-7Rα may be essential for NK T cell development as well. Because IL-7 deficiency does not affect NK T cell development [163], both IL-15 and thymic stromal cell lymphopoietin (TSLP); whose receptor complex includes IL-7Rα in common with IL-7 [164] may act synergistically to signal NK T lymphopoiesis as well as in their homeostasis in vivo.

Models and pathways

The signals required for β selection are absolutely essential for NK T cell lymphopoiesis [165]. Thus signalling through the TCRβ-pre-Tα complex involving the ζ chain of CD3 [110], Lck [166], Fyn [166, 167] and Tcf1 [168] is important at this stage of NK T cell ontogeny (fig. 6). The development of NK T cells depends on CD1d [28–30]. CD1d is expressed predominantly by the CD4+8+ double-positive cells of the thymus [19]. Therefore, in contrast to positive selection of the mainstream TCR repertoire by MHC class I and class II molecules expressed by the thymic epithelium [20], positive selection of the Vα14Jα15-positive NK T cells is accomplished by interactions with CD4+8+ thymocytes [19]. In this regard, it is interesting to note that positive selection of NK T cells does not require the Ras/Mek signalling pathway [169]. Positive selection of mainstream T cells, on the other hand, requires signal transduction down the Ras/Mek pathway [169]. Thus, the signalling pathway for the positive selection of NK T cells is critically different from that of mainstream T cells.

As described above, the expression of the TCR β chain of NK T cells requires pre-Tα, i.e. for β selection [165]. Together with the fact that CD4+8+ thymocytes are required for positive selection of NK T cells, the ontogeny of this subset of T cells must proceed through the same lineage pathways as mainstream T cells (fig. 6). Nevertheless, the fact that NK cells and $\gamma\delta$ T cells differentiate earlier than the $\alpha\beta$ T cells and because the NK T cell developmental programme closely matches that of NK cells, commitment to the NK T lineage might occur prior to that of mainstream $\alpha\beta$ T cells. Future experiments with precursor transfer and/or gene-deficient mice should help resolve whether the NK T lineage follows a distinct developmental pathway or that of mainstream T lymphocytes.

NK T cells also undergo negative selection. One model to study this process consisted of neonatal administration of staphylococcal enterotoxin B (SEB). SEB efficiently deletes Vβ8+ mainstream T cells [170]. Neonatal administration of SEB also deleted Vβ8+ NK T cells [113]. The expression of an endogenous antigen Mls-1^a deletes Vβ8.1+ mainstream T cells in vivo during thymic ontogeny [170]. However, the expression of Mls-1^a did not significantly alter the development of Vβ8.1+ NK T cells [113]. Why the two thymocyte subsets respond differentially to Mls-1^a has not yet been clarefied. The TCR α chain with which Vβ8.1 pairs and the antigen-presenting molecule, MHC class II versus CD1d1, that interacts with the TCR may be critical factors that influence how Mls-1a negatively selects Vβ8.1+ mainstream T cells but not NK T cells. Negative selection is also observed in mice that constitutively express a CD8$\alpha\beta$ transgene from the heterologous CD2 promoter [16]. Such animals express CD8 from the immature TCR$^{-/low}$CD8+ stage onward to the mature CD4+ and CD8+ single positive stage (see fig. 6) [171]. The CD8$\alpha\beta$ transgenic animals developed TCR^{int} NK1+ NK T cells but had lost the expression of the skewed TCR repertoire; i.e. only a small fraction of the NK T cells expressed Vβ8, Vβ7 and Vβ2 TCR β chains but instead expanded the repertoire expressing the Vβ6 TCR β chain [16]. Together, the studies with neonatal administration of SEB as well as transgenic mice that constitutively express CD8$\alpha\beta$ provide compelling evidence for negative selection within the NK T cell compartment.

Yet another question remains, and that is whether a certain precursor commits to NK T cell lineage by specifically rearranging the Vα14 and Jα15 gene segments which upon productive rearrangement beget the NK T lineage – *the instructive model*. The competing model would predict that akin to mainstream T cells, *Tcr* gene segments rearrange randomly and the productive ones are subjected to positive and negative selection by CD1d1 and beget the NK T lineage – *the stochastic model*.

Nucleotide sequence analyses of the V and J junctions within rearranged Vα14Jα15 genes revealed that the canonical TCRα sequence of the CD1d1-reactive NK T cells is put together by random events that also include N nucleotide additions [133, 172, 173]. Moreover, the second allele also showed evidence of random rearrangement within the *Tcr* locus [133, 172, 173]. More recently, genomic DNA sequence analysis of NK T cell-derived Vβ8.2 transgenic thymocytes prior to selection (TCR low thymocytes) revealed random rearrangement of Vα14 with Jα15 segments resulting in a high frequency of non-canonical Vα14+ TCR α chain [173]. The frequency of the canonical Vα14Jα15+ TCR α chain containing the canonical sequence increased within the Vβ8.2 transgenic thymocytes following positive selection (TCR^{int} thymocytes) by CD1d1 [173]. Together, these data support the stochastic model for thymocyte commitment to the NK T lymphocyte lineage.

In summary, Vα14Jα15-positive NK T cells develop in the thymus where their TCR repertoire is moulded by both positive and negative selection processes. The developmental pathway that begets NK T cells and mainstream T lymphocytes during thymic ontogeny seems very similar. However, the signals required for NK T cell ontogeny, although in many ways matching those required for

mainstream T cell development, in some critical ways differ from the latter and overlap signals required for NK cell development.

NK T cell function

The physiological role of NK T cells, *albeit* elusive, is thought to be immunoregulatory in nature [reviewed in ref. 5], a function controlled by CD1d [22, 87]. They are amongst leukocytes that secrete large amounts of IL-4 without prior priming with this cytokine [12]. IL-4 is an immunomodulatory cytokine that can polarise newly activated CD4+ Th cells towards Th2 effector function [3]. Their ability to secrete IL-4 promptly in response to primary stimulation and hence their potential to polarise CD4+ T cells towards a Th2 immune response has aroused interest concerning their role in infection and immunity. NK T cells are also implicated in the control of autoimmune responses. What emerges from studies focused on the role of NK T cells in autoimmune diseases is that they elaborate an IL-4-mediated immunoregulatory role in vivo. Additionally, NK T cells are known to mediate tumour immunity by virtue of their ability to secrete IFNγ themselves or to induce this activity in NK cells. Studies on the mechanism(s) by which NK T cells function in tumour immunity have led to important insights into their physiological role.

Cytokine elicitation and functional consequences

The first recognised function of freshly isolated NK T cells was their ability to elicit both Th1 and Th2 cytokines *in vitro* in response to a primary stimulus, without need for priming with the elaborated cytokines. The elicited Th1 cytokines include IFN-γ and tumour necrosis factor-β (TNF-β) and the Th2 cytokines include IL-4, IL-5 and IL-10 [87, 117, 174–176]. More recently, the secretion of other pro-inflammatory factors such as GM-CSF, lymphotactin, macrophage inflammatory protein (MIP)-1α and MIP-1β has also been attributed to NK T cells (table 3) [87, 177]. Nevertheless in vivo stimulation of NK T cells by cross-linking their TCR with anti-CD3ε monoclonal antibody results in the rapid, i.e. within 60–90 min, secretion of large amounts of IL-4 [12, 178–180]. IL-2 and IFN-γ are also elicited by such polyclonal activation of T cells in vivo [12]. The prompt secretion of IL-4 was attributed to NK T cells because both β_2m- and CD1d1-null mice lacking NK T cells were deficient in this function [28–30, 179]. Additionally, α-GalCer activates NK T cells in vivo resulting in the secretion of both IL-4 and IFN-γ [13, 14]. Thus the cytokine elicitation pattern of NK T cells seems to resemble that of Th0 cells. A recent study [177] focused on gene regulation in activated NK T cells revealed that transcription factors that bestow a Th2 phenotype upon CD4+ cells were upregulated within an IL-4-secreting NK T cell clone (described below). Thus NK T cells may be hardwired during development and differentiation for prompt secretion of large amounts of IL-4 upon in vivo activation.

Akin to mainstream T cells, activation of NK T cells requires signals from the TCR as well as CD28. The presence of soluble CTLA4 during intravenous anti-CD3ε administration inhibits the activation of NK T cells [12]. Thus, the second signal emanating from the interactions of CD28 expressed by the T lymphocyte with CD80 and/or CD86 expressed by the antigen-presenting cell is important for NK T cells activation. Following activation in vivo with anti-CD3ε, hepatic NK T cells rapidly, within 2 h, die [181]. Rapid loss of the large majority of NK T cells is observed in vivo following activation by the antigen α-GalCer [146]. Their death following in vivo stimulation by anti-CD3ε is attributed to apoptosis because a large majority of the activated NK T cells express Annexin V [181, 182]. NK T cells regenerate within the next couple of days. Regeneration requires proliferation, which occurs within the bone marrow [181]. This process of activation-induced cell death and the regeneration of NK T cells from the bone marrow are mimicked by IL-12 [181]. IL-12 mediated death of hepatic NK T cells could then explain how *L. monocytogenes* and *M. bovis*, both of which are potent inducers of IL-12, down-regulate the NK T cell-mediated inflammatory process within the liver of infected mice [183–185]. Thus shortly upon executing their function, NK T cells die, a feature they share with other cells of the innate immune system.

The physiological relevance of pro-inflammatory and anti-inflammatory cytokine secretion by activated NK T cells has been addressed in numerous experimental systems. The first of these predicted that IL-4 secreted by NK T cells by in vivo stimulation may play a role in Th2 differentiation and hence in type 2 immune responses to antigens. Previous studies had established that administration of goat anti-immunoglobulin-D (IgD) into mice resulted in the elicitation of IgG1 and IgE antibodies. In this system, IgG1 and IgE production requires CD4+ T lymphocytes and IL-4 [12, 180]. How anti-IgD stimulates IgG1 and IgE production in vivo remains unclear. However, β_2m-null mice, deficient amongst other things in NK T cells, do not respond to anti-IgD-mediated stimulation and hence do not secrete IgE. These results implicated CD1d1-restricted NK T cells in the anti-IgD-mediated IgE response [178, 179]. Contrarily however, CD1d1 and CD1d2 double-knockout as well as CD1d1-deficient mice, both of which have intact mainstream T cells but lack NK T cells, were perfectly capable of eliciting an anti-IgD-mediated IgE response [28–30]. Moreover, the loss of anti-IgD-mediated IgE response in $\beta_2m^{0/0}$ mice is due to FcRn- (a β_2m-dependent molecule) mediated rapid catabolism of injected antibody [186, 187]. Thus NK T cells do not play a role in the anti-IgD-induced IgE response in vivo.

Infection and immunity

Adaptive immunity against infectious agents such as bacteria as well as protozoan and helminthic parasites depends on the functions of CD4+ T lymphocytes. The kind of CD4 response, whether Th1 or Th2, to the infectious agent is dictated by a variety of factors of which the cytokine milieu during the time of T cell priming by antigen plays a critical role in the outcome. Because NK T cells have the potential for eliciting Th1 and Th2 cytokines promptly upon in vivo activation, they are implicated in the Th1 versus Th2 immune responses to bacterial and parasitic infections.

A comprehensive analysis was undertaken using soluble antigens and infectious agents that are known to elicit Th2 responses to determine the role of NK T cells in this polarised immune outcome [188]. Various parameters, IL-4 and IgE response, eosinophilia, as well as airway hypersensitivity, were monitored following antigen, soluble and particulate in the form of the parasite, delivery through various routes, subcutaneous, airway and intestinal, into wild-type and β_2m-deficient mice. Note, the latter are incapable of promptly secreting IL-4 in response to intravenous administration of anti-CD3ε. The data revealed no difference in Th2 response between wild-type and β_2m-deficient mice to soluble keyhole limpet haemocyanin, aerosolised ovalbumin, embolisation with *Schistosoma mansoni* eggs, infection with metacyclic promatigotes of *Leishmania major* or intestinal infection of *Nippostrongylus brasiliensis* [188]. *L. major* does indeed induce IL-4 early during infection, but is secreted by a non-NK T cell subset [189, 190].

However, intraperitoneal infection of mice with *S. choleraesuis* or *L. monocytogenes* results in the recruitment of NK T cells to the peritoneum [129, 130]. The *Salmonella*-recruited NK T cells in the peritoneal exudate secrete IL-4 [129]. The elicited IL-4 down-regulates IL-12 production and hence negatively controls the generation of the Th1 immune response. Most importantly, the regulatory role of IL-4 over IL-12 was abolished in Jα15- (i.e. Jα281, the TCR *J*α gene segment utilised by Vα14-positive NK T cells) deficient mice resulting in aberrantly high levels of IL-12 [129]. Thus, NK T cells may play significant role in the early phase of infection that in turn influences adaptive immunity to intracellular bacterial infections.

Additionally, a role for NK T cells has been implicated in eliciting immune responses to other infectious agents. For example, *Salmonella* spp. [129], *L. monocytogenes* [183, 184], *M. tuberculosis*, *M. bovis* (bacillus Calmette Guerin, BCG) [191], *Toxoplasma gondii* [192, 193] and *L. major* [194] in resistant strains of mice elicit Th1 immunity [reviewed in ref. 195]. In these cases, the ability of NK T cells to secrete IL-2 or IFN-γ themselves or to induce this activity in NK cells (described below) is exploited. In the case of *T. gondii*, CD8+ T cell function is recruited in the absence of CD4+ T lymphocytes that are normally required for IFN-γ-mediated Th1 immunity. Further analysis revealed a role for IL-2 released by NK T cells in recruiting CD8+ T cell-mediated immunity to *T. gondii* in the absence of MHC class II-restricted T cells [193].

Human CD1b- and CD1c-restricted presentation of mycobacterial lipid and glycolipid antigens is well documented [reviewed in ref. 31]. However, immunity to mycobacteria in mice, which express only the CD1d isotype, suggests that CD1d may not be directly involved in antigen presentation [196, 197]. Nevertheless a recent study revealed that NK T cells contribute to the formation of mycobacterial cell wall-induced granulomas [198]. PI-mannosides were tentatively identified as the causative antigen in this granulomatous reaction in wild-type mice. Because this reaction was absent in the Jα15-deficient mice, granuloma formation involved NK T cells and suggests CD1d-mediated antigen presentation [198]. The response of CD1d-deficient mice to mycobacterial cell wall glycolipids has yet to be determined. In this context it is interesting to note that whether or not direct antigen presentation by CD1d is involved, *M. bovis* infection results in the disappearance of hepatic NK T cells, presumably due to the induction of IL-12 and IL12-mediated NK T cell death [191]. Inoculation with BCG altered the density of NK T cells; the altered density correlated with an IFN-γ-secreting phenotype [191]. It is possible that the IFN-γ-secreting cells were NK cells and not NK T cells. The IFN-γ elicited in response to BCG may be involved in the mycobacterial cell wall-induced granuloma reaction.

Although infection by *Plasmodium* spp. gets the better of some of us, resistant individuals elicit both cellular and humoral responses to the malarial vector [199]. The role of CD1d and NK T cells in immunity to the malarial vector has recently been a subject of much interest for it offers the possibility for vaccine development in the face of a rapid evolution of drug-resistant variants of *Plasmodium* spp. [199]. Experimental infection of mice with sporozoites of *Plasmodium yeolii* results in the expansion of hepatic NK T cells [200]. In vitro co-culture studies using the activated NK T cells generated during the acute phase of infection revealed that these lymphocytes inhibit growth of the liver stage parasite [200]. Moreover, the observed anti-*Plasmodium* effector function requires IFN-γ [200]. In another study, activation of NK T cells in vivo with the NK T cell antigen α-GalCer 1 or 2 days prior to infection with *P. yoelii* sporozoites conferred immunity to the liver-stage parasite [201]. Akin to the in vitro findings, the anti-plasmodial effector function in vivo was primarily mediated by IFN-γ. Nevertheless, surprisingly, this IFN-γ response requires neither IL-12 nor NK cells, but IFN-γ secreted by NK T cells sub-serves the anti-plasmodial effector function of NK T cells [201]. Additional data revealed that the effector function of NK

T cells against malarial sporozoites did not require Fas ligand, perforin or TNF-α [201]. Thus NK T cells play an important role in the early immune response to plasmodial sporozoites in mice. Whether α-GalCer-activated NK T cells elicit early immunity to sporozoites in humans needs study.

NK T cells have the potential to bias antigen-specific Th immune responses toward the Th2 type which results in providing help to B cells for antibody elicitation. Two groups have studied the role of NK T cells in the anti-plasmodial antibody response. Results from one group revealed a GPI-specific CD1d-restricted and NK T cell-dependent antibody response to plasmodial sporozoite-derived circumsporozoite protein [202]. In contrast the results of the second group revealed an MHC class II dependence but CD1d and NK T cell independence in anti-plasmodial humoral immunity to the same protein [88]. The basis of these contradictory results remains unclear.

Tumour immunity

NK cells as well as the type I cytokines IFN-γ and IL-12 play an important role in immune surveillance against tumour growth and metastasis. Recent studies have revealed that an important function of NK T cells in vivo is host protection against tumour growth and metastasis [67, 203, 204]. This immune surveillance function of NK T cells requires IL-12 or its induction by NK T cell stimulation in vivo by α-GalCer [67, 205]. Additionally, immunity to chemically induced tumours is conferred by NK T cells without the need for exogenous IL-12 or α-GalCer [204]. Thus a role in immune protection against spontaneously arising tumours and tumour metastasis is ascribed to NK T cells in vivo.

Their role in immune surveillance in vivo has aroused interest in the mechanism of action of NK T cells. α-GalCer, the potent synthetic glycolipid antigen presented by CD1d, activates NK T cells in vivo resulting in the secretion of both IFN-γ and IL-4 [13, 14, 69]. Both cytokines are elicited promptly in response to α-GalCer; however, IFN-γ is maintained only acutely while IL-4 is maintained chronically [13]. The chronic maintenance of IL-4 in the milieu of differentiating Th cells polarises them towards a Th2 phenotype [13]. Moreover, the IFN-γ response to α-GalCer seems to be elicited by NK cells requiring IL-12-mediated activation [67, 68, 206]. IL-12 is a pro-inflammatory cytokine elicited by activated myeloid cells such as dendritic cells and macrophages [7]. How activation of NK T cells by α-GalCer results in IL-12 production is discussed below [207].

Whether NK T cells directly mediate cytotoxic activity on target tumour cells is controversial. All data that support NK T-mediated tumour-specific cytolytic activity are derived from in vitro studies [208–211]. It is clear, however, that the in vivo activation of NK T cells lead to the induction of IL-12 [181]. IL-12, as discussed above, induces NK T cell death within a few hours [181] and may not be available for tumour-specific cytocidal activity in vivo. The ability of activated NK T cells to promptly activate effector cells of the innate and adaptive immune system, particularly NK cells [13, 14, 212] and CD8+ T lymphocytes [13, 14, 213] may be responsible for the observed NK T-mediated cytolytic activity in vivo.

Neverthelesss in vivo NK T cell-mediated cytotoxicity has been observed in a murine model for hepatitis [214, 215]. Hepatitis in this model is induced by intravenous administration of concanavalin A (Con A) into mice. Neither CD1d1- nor Ja15-^{1}-deficient mice develop experimental hepatitis upon Con A treatment [214, 215]. Furthermore, adoptive transfer of Con A-activated NK T cells into CD1d1-deficient mice transferred the disease [215]. Additionally, Con A-treated mice that only develop NK T cells by virtue of transgenic expression of the mouse *Va14Ja15/Vb8.2* genes [69] also develop hepatitis. Thus, NK T cells are implicated in the development of autoimmune hepatitis. Furthermore, analysis of the killing mechanism revealed that the activation of NK T cells required IL-4 [214], and that activation induced granzyme B [214] as well as Fas L [214, 215] expression. Neither perforin- [214] nor *gld/gld*- [214, 215] deficient mice are affected by Con A stimulation in vivo. Thus perforin-granzyme B and Fas and Fas L are implicated as molecular effectors of NK T cell-mediated cytotoxicity in vivo.

Autoimmunity

Several lines of evidence suggest a link between NK T cell deficiency or dysfunction and autoimmune diseases [216–224]. The best studied of these disorders is insulin-dependent diabetes mellitus (type I diabetes). Both humans and mice predisposed to or afflicted by type 1 diabetes have fewer NK T cells and those present are dysfunctional, in that they are unable to secrete IL-4 promptly in response to cross-linking of their antigen receptors [219, 221, 223]. In non-obese diabetic (NOD) mice, the mouse model for human type 1 diabetes [225, 226], adoptive transfer of thymic NK T cells into pre-diabetic animals corrected the deficiency and the disease [222]. Protection from disease by adoptive transfer of NK T cells required IL-4 and/or IL-10 [222]. Moreover, transgenic expression of Vα14Jα15 TCR in NOD mice also protects them from diabetes [227]. Additionally, administration of recombinant IL-4 itself [228] to pre-diabetic NOD animals prevented them from developing diabetes. Thus an IL-4-mediated immunoregulatory role in vivo is ascribed to NK T cells.

To define the molecular basis for NK T cell dysregulation in diabetic individuals, a comprehensive analysis of the mRNA expression profile of human NK T cell clones iso-

lated from monozygotic twins discordant for type 1 diabetes and IL-4 secretion was undertaken using gene chips. One clone was isolated from the normal twin; it elicits IL-4 upon activation through its antigen receptor. The other clone was derived from the former's identical, but diabetic sibling; it is unable to secrete IL-4 (IL4-null) upon TCR-mediated activation. The results suggest that the NK T cells from the diabetic twin had dysregulated genes and were thus polarised towards a Th1 phenotype. This gene expression pattern is distinct from the non-diabetic twin's NK T cells, which expressed a pattern reflective of a Th2 phenotype.

Specifically, of the ~1500 genes expressed within the two NK T cell clones detectable under resting and activated conditions, 226 and 86 transcripts were significantly modulated within the IL-4-secreting and IL-4-null NK T cell clones, respectively, upon activation. Of the modulated transcripts, only 28 were shared between the two clones. Interestingly, the expression of transcription factors and signalling molecules were amongst those differentially regulated between the two NK T cell clones. Transcription factors that bestow a Th2 phenotype upon CD4+ cells (e.g. GATA3 and JunB) [229, 230] were up-regulated in the activated IL-4-secreting clone. In contrast, signalling molecules (e.g. STAT1, STAT4 and CD161) involved in transducing Th1 cytokine signals (e.g. IFN-γ and IL-12) [231–234] were up-regulated in the IL-4-null clone. NFAT4, a transcription factor thought to suppress IL-4 transcription [235], was over-expressed in the IL-4-null clone, consistent with its inability to secrete IL-4. These differences explain why normal NK T cells promptly secrete large amounts of IL-4 upon in vivo activation. Additionally, they also explain how NK T cells in diabetics may be dysregulated and attain a Th1 phenotype.

Cross talk between cells of the innate and adaptive immune system

The discovery of α-glycosylceramide as a synthetic NK T cell antigen has prompted studies of how NK T cells function *in vivo* using the α-anomeric glycolipid as probes. Such studies have revealed that α-GalCer can stimulate NK T cell function *in vivo*. The activation of NK T cells *in vivo* results in the prompt secretion of IL-4 and IFN-γ [13, 14]. Their ability to secrete IFN-γ is thought to mediate the anti-tumour activity of α-GalCer described above [14, 212]. However, careful examination of the cell type that secretes IFN-γ in vivo in response to α-GalCer revealed NK cells as the major source of this type I cytokine [14, 212]. Additionally, the ability to induce IFNγ secretion by α-GalCer requires IL-12 [68, 206, 236]. These findings raise the question as to how the activation of NK T cells results in the secretion of IFN-γ by NK cells. The gene expression studies described above with the IL-4-secreting NK T cells derived from the non-diabetic individual revealed an interesting possibility. Activation of the IL-4-secreting NK T cells results in the transcription of high levels of messages for cytokines/chemokines important for the recruitment, activation and differentiation of macrophages and dendritic cells (table 3). The pro-inflammatory factors such as GM-CSF, MIP-1α and MIP-1β promote the recruitment, activation and/or differentiation of dendritic cells and macrophages (fig. 7) [237, 238]. Activated dendritic cells and macrophages secrete IL-12 and/or other factors that stimulate NK cells and T cells to secrete IFN-γ [7], explaining how α-GalCer induces IFN-γ secretion by NK cells in an IL-12-dependent manner.

Prompt IL-4 secretion upon stimulation of NK T cells in vivo by α-GalCer also has functional consequences. IL-4 activates B cells as judged by the expression of the early activation marker CD69 [239]. Expression of CD69 is induced within 6 h of α-GalCer treatment [14]. Activated B cells are also induced to express CD86, the ligand for the T cell co-simulatory molecule CD28 [13]. Within 5–6 days of administration, α-GalCer also induces IgE secretion [13, 15], a hallmark of type 2 immune response. Additionally, the stimulation of NK T cells by α-GalCer at the time of immunisation with nominal antigen results in an antigen-specific IgE antibody response [13]. Thus, IL-4 secreted by activated NK T cells does indeed have a Th2 polarising effect in response to antigen in vivo.

α-GalCer-activated NK T cells cross-talk with CD8+ T lymphocytes [213]. Such communication between NK T cells and CD8+ T lymphocytes is absent in CD1d1- and Jα15-deficient mice. Activation of CD8+ cells required CD1d1 for the presentation of α-GalCer as well as CD40-CD40L interactions between NK T cells and dendritic cells. CD8+ lymphocytes thus activated possess the ability to secrete IFN-γ as well as mediate cytotoxicity. In the presence of a tumour, specific cytotoxic T lymphocytes were elicited rapidly [213]. Thus, NK T cells activated by α-GalCer in vivo can communicate with CD8+ T lymphocytes as well.

Thus in summary NK T cells upon activation *in vivo* promptly secrete numerous cytokines that include pro-inflammatory and anti-inflammatory factors (table 3) without the need for prior sensitisation to these factors. The prompt elicitation of cytokines/chemokines in response to antigen probably helps jump-start the innate and adaptive components of the immune system (fig. 7). The Th1-inducing cytokines produced for a short period and IL-4 secretion sustained for a longer period have the potential to stimulate both the cellular and humoral limbs of the adaptive immune system. However, the IL-4 burst upon activation of NK T cell by antigen counter-regulates Th1-promoting cytokines, and helps Th2 differentiation and further elaboration of IL-4; this sustains IL-4 in vivo in a chronic manner and hence promotes Th2-mediated immunity.

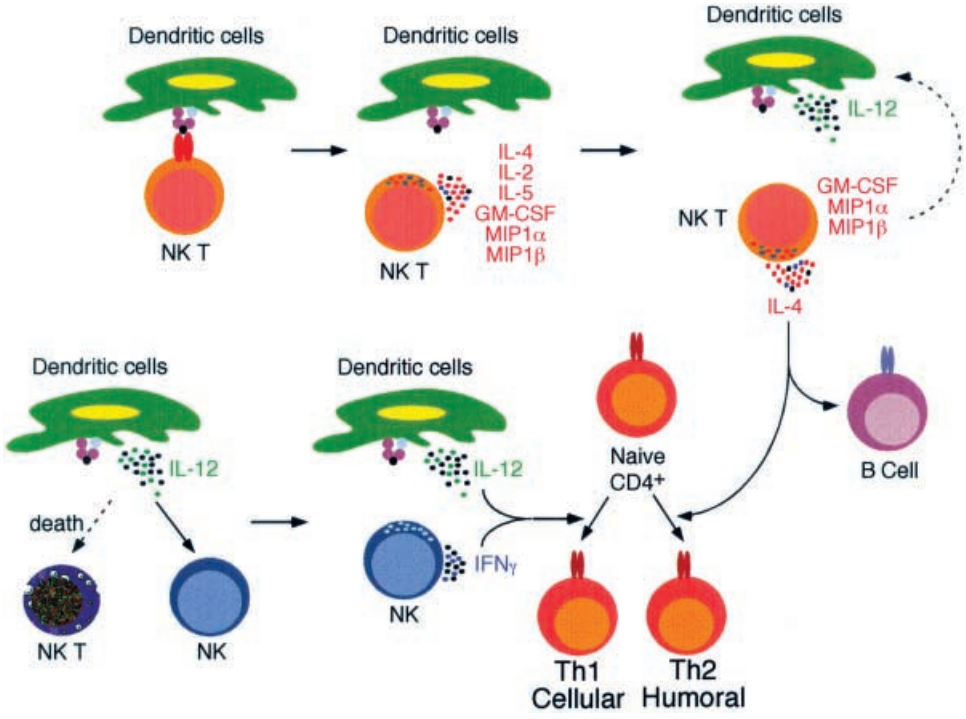

Figure 7. The predicted immunological role of NK T cells. NK T cells participate in cross-talk between members of the innate and the adaptive immune system by deploying cytokine/chemokine messengers. Current data suggest that NK T cells upon activation *in vivo* promptly secrete several cytokines/chemokines (see table 3). Of these, IL-4 activates B cells *in vivo* resulting in their differentiation to antibody-secreting plasma cells. IL-4 can also skew the differentiation of CD4+ T cells towards a Th2 phenotype. GM-CSF, MIP-1α and MIP-1β facilitate the recruitment, activation and differentiation of macrophages and dendritic cells resulting in the production of IL-12 and possibly other factors. IL-12, in turn, stimulates NK cells to secrete IFN-γ on the one hand, but induces cell death of NK T lymphocytes on the other. Along with IL-12, IFN-γ can polarise the differentiation of antigen-activated CD4+ T cells towards a Th1 phenotype. Thus IL-4, GM-CSF, MIP-1α and MIP-1β can be thought of as primary cytokines of NK T cells, and IL-12 and IFN-γ as secondary cytokines. The Th1 cytokines can counterbalance Th2 and vice versa. How this is accomplished under conditions whereby both Th1 and Th2 cytokines elicited by NK T cells are present simultaneously remains unclear (see text for references).

How CD1d controls NK T cells function: a hypothesis

Immunity to infectious agents is accomplished in two phases; it is initiated by the recognition of antigen, which is followed by a response. As has been shown, CD1d controls the recognition phase and NK T cells elicit the response. The control of the NK T cell response by CD1d depends on its function as a glycolipid-presenting molecule. The recognition of the glycolipid-presented by CD1d to NK T cells depends on the nature of the TCR expressed by the effector cells. The naturally processed antigen(s) recognised by NK T cells remains elusive. Consequently, the best available glycolipid to study the CD1d-NK T cell system is the synthetic antigen α-GalCer. On the basis of current knowledge of CD1d-NK T cell biology, the following can be surmised regarding the naturally processed NK T cell antigen. (i) Despite the fact that GPI is the natural ligand of mouse CD1d1, it is Iess likely that either GPI or PI is the NK T cell antigen. Although PI can serve as an antigen for a few NK T cells [74], neither PI nor GPI stimulates the large majority of NK T cells [88]. (ii) An α-anomeric glycolipid may be the elusive natural NK T cell antigen because none of the commercially available β-anomeric glycosylceramides stimulate NK T cells [69]. (iii) α-anomeric glycolipids are rare in the animal kingdom, and certainly have not been found to date in mammals. Recognition of infectious agents by antigen-presenting cells is predicted to induce the transient synthesis of an α-anomeric glycolipid, which when presented by CD1d stimulates NK T cells. (iv) A single neo-self antigen is predicted to control NK T cell function. (v) *Albeit* controversial, because the highly conserved TCR α chain pairs with modest numbers of TCR β chains that have highly diversified CDR3β regions, numerous antigens are thought to function in NK T cell activation. Nevertheless because there are numerous ways by which multiple TCRs might interface a single antigen, diversity within the NK T cell receptor β-chain does not necessitate recognition of multiple antigens. Such a recognition process is not unusual, in that a single MHC-restricted peptide epitope elicits mainstream T lymphocytes that express diverse TCRs indicating numerous ways of recognising a single antigen [240].

The activation of NK T cells in the absence of microbe-derived antigen presentation by CD1d is noteworthy [10,

87, 88, 94]. NK T cells function in immune surveillance against spontaneously arising tumours that do not express CD1d [67, 204]. Additionally, NK T cells are quiescent in vivo in the absence of any infectious stimuli. For these reasons, the induced nature of the NK T cell antigen is postulated. Recently, a role for CD1 in monitoring membrane integrity has been evoked because it binds and presents lipids and glycolipids to specific T cells [241]. In keeping with this hypothesis, infectious assaults, such as viral and bacterial infections as well as glucose starvation, are speculated to induce unfolded protein response [reviewed in ref. 242]. An unfolded protein response increases cytosolic inositol levels, a key to the induction of membrane biogenesis [243]. Inositol plays an important role in regulating phospholipid biosynthesis [244] and hence in membrane biogenesis [243, 245]. Phospholipids and glycolipids play an important structural role in maintaining membrane integrity. Thus the induction of the unfolded protein response may include the biosynthesis of neo-self lipids that are normally not encountered by NK T cells. Encounter with the neo-self glycolipid, the elusive α-anomeric glycosylceramide, turns on NK T cells. Because the products of the unfolded protein response are similar irrespective of the cause, the induction and presentation of a common antigen are postulated during viral or bacterial infections or glucose starvation. A virtue of the unfolded protein response relevant to the CD1d-NK T cell antigen presentation and recognition system is its rapidity and the short half-life (about 2 min!) of the induced products [reviewed in ref. 242]. This might explain the rarity of the NK T cell antigen and hence why it has eluded identification.

Thus CD1d functions as a sensor, sensing alterations in cellular lipid content by virtue of its affinity for such ligands. The presentation of a neo-self glycolipid, presumably induced by infectious assault of antigen-presenting cells, activates NK T cells. Activated NK T cells promptly release pro-inflammatory and anti-inflammatory cytokines and jump-start the immune system. Shortly thereafter, NK T cells are lost to death but replenished over the next couple of days. Identification of the elusive natural antigen of NK T cells will shed more light on how the CD1d-NK T cell system works in vivo, especially with regard to antigen expression and presentation to this unique T lymphocyte. The significance of NK T cell function in vivo, established with α-GalCer, in infection and immunity, in tumour immunity as well as in autoimmunity has been demonstrate, but most important, the NK T cell antigen will tell us whether the CD1d-NK T cell system has any physiological importance.

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