The unique role of natural killer T cells in the response to microorganisms

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Abstract | Natural killer T (NKT) cells combine features of the innate and adaptive immune systems. Recently, it has become evident that these T cells have crucial roles in the response to infectious agents. The antigen receptor expressed by NKT cells directly recognizes unusual glycolipids that are part of the membrane of certain Gram-negative bacteria and spirochetes. Moreover, even in the absence of microbial glycolipid antigens, these T cells respond to innate cytokines produced by dendritic cells that have been activated by microbes. This indirect sensing of infection, by responding to cytokines from activated dendritic cells, allows NKT cells to react to a broad range of infectious agents.

Natural killer T (NKT) cells have been found to influence diverse immune responses, especially in mice, including the maintenance of self-tolerance and the surveillance for tumours[1]. Homologous populations of NKT cells have been found in rodents and primates[2], and although this evolutionary conservation indicates that NKT cells have a distinct role in immunity, their precise physiological function has eluded definition. Several recent studies, however, indicate that NKT cells could have been selected in evolution primarily for their role in antimicrobial defence.

NKT cells were originally named because of the co-expression of a T-cell receptor (TCR) along with typical surface receptors for NK cells[2,3]. This co-expression perfectly illustrates the hybrid nature of these cells. NK cells are part of the innate immune system, as they effect rapid killing and cytokine responses without the need for extensive cell division or differentiation. By contrast, conventional T cells are, together with B cells, the prototypic cell types of adaptive immunity. Adaptive immune cells express highly diverse antigen receptors formed by somatic recombination of the variable (V) and joining (J) gene segments encoding them. Although one lymphocyte expresses only a single rearranged antigen receptor gene, the diversity in the population confers on lymphocytes the capacity to recognize an almost infinite number of different antigens. Responses mediated by B and T cells, therefore, require several days to clonally expand those rare cells that express a particular antigen-specific receptor. Following a second exposure to the antigen, these antigen-specific clones exhibit a more rapid and vigorous response — the hallmarks of immune memory.

Conventional T cells recognize peptides presented by or bound to cell-surface proteins encoded in the major histocompatibility complex (MHC). T cells were initially divided into two categories (FIG. 1a,b): those that express CD4 on their cell surface ($CD4^+$ T cells) recognize peptides presented by MHC class II molecules (FIG. 1a), secrete cytokines and are known as 'helpers' because they regulate or 'help' the responses of other cell types; $CD8^+$ T cells recognize peptides that are presented by MHC class I molecules (FIG. 1b), and although they can secrete cytokines, they are also potent killers of cells that present antigenic peptides.

We now recognize a third category of T cell, which recognizes antigens presented by CD1 molecules (FIG. 1c). These cells can, but do not necessarily, express CD4 or CD8. In humans and most other mammals, four CD1 genes (*CD1A–D*) encode antigen-presenting proteins[4]; mice and rats have *Cd1d* only. The analyses of crystal structures show that CD1 molecules have an MHC-type antigen-binding groove that is bordered by α-helical domains[5] (FIG. 2). MHC class I and class II molecules are encoded by highly polymorphic genes that are closely linked. By contrast, CD1 genes are encoded outside the MHC, and with few alleles, they are nearly invariant. Moreover, CD1 molecules have a deeper, narrower and more hydrophobic antigen-binding groove than their MHC-encoded counterparts[4,5]. The CD1 groove is adapted for the presentation of lipid antigens, which are mostly glycolipids, instead of peptide presentation. CD1 molecules traffic through vesicles of the endolysosomal system[4], where they bind the lipid antigens that will be presented on the cell surface. Once bound to CD1, polar head groups of the lipid antigen point upwards from the

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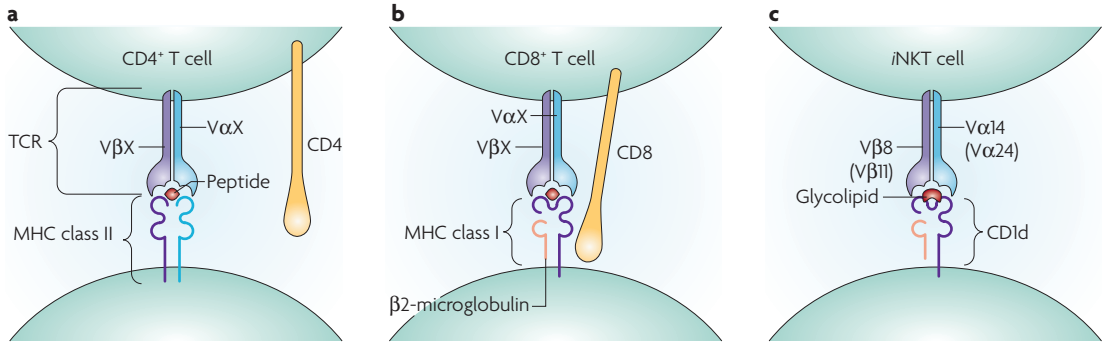

Figure 1 | **Antigen recognition by T cells.** The three main types of antigen recognition by T cells that express T-cell receptors (TCRs) composed of α- and β-chains. **a** | Recognition of peptides that are presented by major histocompatibility complex (MHC) class II molecules by CD4⁺ T cells. **b** | A CD8⁺ T cell recognizing its antigenic peptide presented by MHC class I molecules. **c** | Invariant natural killer T (*i*NKT) cells are a third category of T cell, and can be CD4⁺, CD8⁺ or double negative for these surface markers and recognize lipid antigens presented by CD1 molecules. The 'X' indicates variable antigen-receptor chains. CD8 interacts with a non-polymorphic portion of the MHC class I molecule, whereas CD4 interacts with a non-polymorphic part of the MHC class II molecule.

middle of the CD1 groove (FIG. 2), and the TCR probably recognizes these polar head groups together with α-helical regions of the CD1 molecule.

The concept that CD1 molecules present microbial lipids is not a new one. It has been established for some years that the CD1a, CD1b and CD1c molecules present various antigens from the mycobacterial cell wall[4–6]. CD1d, the most distantly related member of the CD1 family of proteins, has been known primarily for its ability to present antigens to a unique population of cells known as NKT cells[2,4]. The precise definition of an NKT cell is not simple, but in this Review we will consider only those NKT cells that recognize CD1d molecules and that have an invariant or canonical TCR α-chain (BOX 1) — here called *i*NKT cells. In mice, the invariant α-chain is encoded by *Vα14* (Vα14*i*), whereas in humans it is encoded by the orthologous *Vα24* (Vα24*i*). For nearly a decade, the only known antigen presented by CD1d and recognized by *i*NKT cells was α-galactosylceramide (α-GalCer) (FIG. 3a). α-GalCer is a synthetic glycosphingolipid (GSL) variant of a closely related molecule originally extracted from a marine sponge in a screen for compounds that could prevent tumour metastases to the liver of mice[7]. It was later found to be a potent agonist for *i*NKT cells[8]. However, α-GalCer was not thought to be a good candidate for the natural antigen because of its unusual α-linkage of the sugar to the ceramide lipid, but it has proved to be a useful tool for dissecting the role of *i*NKT cells in immunity. For example, by using α-GalCer and mouse models deficient in Vα14*i* NKT cells, numerous studies have shown the pivotal roles that *i*NKT cells have in cancer, autoimmunity and inflammatory disease, and in host defence against pathogens[1].

In this Review, we describe the diverse infectious agents, including bacteria, viruses and protozoan parasites, in which *i*NKT cells have been reported to have an important role in the host response; this role is usually a beneficial one that aids clearance of the infectious agent, although in a few cases *i*NKT cells are detrimental. We summarize data indicating that *i*NKT cells respond rapidly to cytokines from dendritic cells (DCs) that have been activated by microorganisms, even when their invariant TCR is not engaged by a foreign lipid antigen, although recognition of a self antigen presented by CD1d might be involved in modulating or initiating this response. Finally, we review the bacterial sources of the glycolipid antigens that engage the invariant TCR expressed by *i*NKT cells, as well as the unique structures of these antigens.

*i*NKT cells in host defence against pathogens

Many studies have been carried out to define the role of Vα14*i* NKT cells in the response to microbial pathogens[9–27]. Most of these used strains that are deficient for Vα14*i* NKT cells. *Jα18*$^{-/-}$ mice[28] specifically lack Vα14*i* NKT cells because they do not have the Jα gene segment required to form the invariant α-chain by rearrangement. The CD1d protein must be engaged by the TCR, otherwise Vα14*i* NKT cells fail to differentiate in the thymus[29–31]; *Cd1d*$^{-/-}$ mice therefore lack Vα14*i* NKT cells, as well as some other CD1d-reactive T cells with more diverse TCRs[32,33]. In some studies, blocking anti-CD1d antibodies were used to assess Vα14*i* NKT-cell function. By these methods, it was shown that Vα14*i* NKT cells participate in the response to various microbial pathogens including bacteria, fungi, parasites and viruses[4,34–36] (TABLES 1,2).

Responses to bacteria. The protective role of Vα14*i* NKT cells was shown in an acute pneumonia model following *Streptococcus pneumoniae* infection[37]. Almost all *Jα18*$^{-/-}$ mice died within several days after intratracheal infection with *S. pneumoniae*, whereas most wild-type mice survived. *Jα18*$^{-/-}$ mice had significantly more bacteria, decreased numbers of neutrophils and lower levels of several cytokines in the lung. Similarly, in pulmonary infection with *Pseudomonas aeruginosa*, it was shown that *Cd1d*$^{-/-}$ mice or mice treated with anti-CD1d antibodies had decreased clearance of bacteria from the lung[38]. The number of neutrophils was decreased in the bronchoalveolar lavage fluid of *Cd1d*$^{-/-}$ mice, and this

correlated with decreased production of a neutrophil chemotactic protein. However, in another study, it was recently reported that *Jα18*$^{-/-}$ mice were not more susceptible to *P. aeruginosa* infection following intratracheal infection[39]. These discrepant observations regarding *P. aeruginosa* might be due to the different strains of bacteria used or the different routes of infection, but they also illustrate the complexity of analysing the role of the relatively small *i*NKT-cell subpopulation, and they reflect controversies that are endemic to the field.

Another example of the importance of Vα14*i* NKT cells is provided by a study of a Lyme disease model system. After intradermal infection with *Borrelia burgdorferi*, *Cd1d*$^{-/-}$ mice developed a greater thickening of the knee and tibiotarsal joints, indicative of arthritis[40]. Bacterial dissemination from skin to urinary bladder was observed in all *Cd1d*$^{-/-}$ mice, but not in most control mice. It has been reported that T cells are not crucial for resistance to *B. burdorferi*[41]; this work, however, was performed on C57BL/6 mice, a background known to be particularly resistant to the development of Lyme disease, and our work in progress (E.T., unpublished observations) indicates an important role for *i*NKT cells *in vivo* when using mice on the BALB/c background.

Responses to parasites. In visceral *Leishmania donovani* infection, it was reported that the parasite burden was significantly higher in the liver and spleen of *Cd1d*$^{-/-}$ mice compared with wild-type mice[42]. *Cd1d*$^{-/-}$ mice showed a defective granulomatous response, which is important for resistance. By using *Jα18*$^{-/-}$ mice, the protective role of Vα14*i* NKT cells also was shown following cutaneous infection with *Leishmania major*[43]. More recently, it was reported that these cells are more important in the response to *L. major* in visceral infection than in cutaneous infection[44], and perhaps this is because Vα14*i* NKT cells are abundant in the liver and spleen.

Following intraperitoneal infection with *T. cruzi*, most of the *Jα18*$^{-/-}$ mice died within a few weeks, and they all developed severe inflammation in the liver, spleen and muscle[45]. The production of cytokines such as interferon (IFN)-γ, tumour necrosis factor (TNF) and nitric oxide (NO) was significantly higher in cultures of spleen cells from *Jα18*$^{-/-}$ mice. These data indicate that mice without *i*NKT cells died from immunopathology as opposed to rampant parasite infection, and that in this experimental system Vα14*i* NKT cells have a beneficial, anti-inflammatory role.

These examples from different parasite infections illustrate the perplexing dual nature of Vα14*i* NKT-cell function. Although in many cases Vα14*i* NKT cells promote microbial clearance, in other cases they might inhibit the synthesis of proinflammatory cytokines, thereby preventing inflammation and decreasing immunopathology.

Responses to viruses. Vα14*i* NKT cells also are involved in the responses to viruses, although unlike bacteria and parasites, viruses contain only host lipids. In a skin infection (zosteriform) model with a virulent strain of herpes simplex virus type 1 (HSV-1), it was shown that *Cd1d*$^{-/-}$ mice are more susceptible to infection, with a more rapid spread of virus to spinal ganglia and delayed virus clearance[46]. Similarly, the virus titres in spinal ganglia were higher in *Jα18*$^{-/-}$ mice than in wild-type mice. In another study, however, *Cd1d*$^{-/-}$ mice were not susceptible to HSV-1 infection[47], although the less virulent HSV-1 strain might account for the different outcome. It was also reported that *Cd1d*$^{-/-}$ mice are more susceptible to genital HSV-2 infection, and that *i*NKT cells are an early source of IFN-γ production following this infection[48].

Several mouse viruses, including vaccinia virus and lymphocytic choriomeningitis virus, downregulate *Cd1d* expression[49,50], suggesting a viral immune evasion mechanism that prevents CD1d-mediated antigen presentation to Vα14*i* NKT cells. This is also true in humans, as Kaposi's sarcoma-associated herpesvirus[51], HSV[52] and HIV[53,54] each use different mechanisms to decrease *CD1D* expression. Additionally, there is a single case report of a girl who died after receiving the varicella vaccine, and the only immune defect characterized was a lack of Vα24*i* NKT cells[55]. Furthermore, boys deficient for the lymphocyte adaptor protein SAP (signalling lymphocyte activation molecule-associated protein), encoded by the

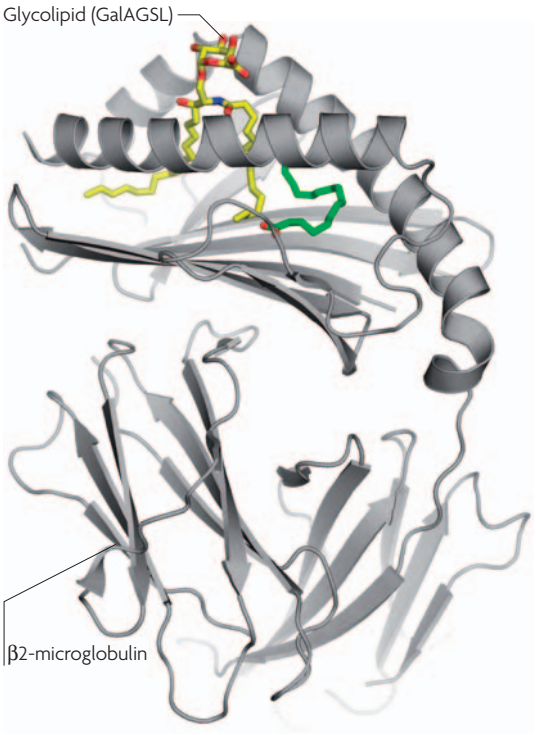

Figure 2 | **Structure of a glycolipid bound to, or presented by, CD1d.** A ribbon diagram of a side view of the mouse CD1d molecule (grey colour). The bound lipid is a synthetic version of the glycosphingolipid from *Sphingomonas yanoikuyae* (yellow backbone). Bound palmitic acid (green) completely fills the CD1d groove. Note how the hexose sugar ring in the horizontal orientation protrudes from the CD1d antigen-binding groove for possible T-cell-receptor recognition. This figure was modified with permission from REF. 101 © (2006) National Academy of Sciences.

Lyme disease
Named after Lyme, Connecticut, USA, where it was described, this disease is found in North America and Europe in some individuals bitten by *Ioxedes* ticks (deer ticks). It consists of a rash and flu-like symptoms in its initial stages, followed by musculoskeletal, arthritic, neurological, psychiatric and/or cardiac manifestations in individuals not treated early on with antibiotics.

Box 1 | What is an NKT cell?

Most of the T cells in mice that express natural killer (NK)-cell receptors also express an invariant Vα14–Jα18 T-cell receptor (TCR) α-chain rearrangement, unlike the highly diverse TCRs expressed by conventional T cells, and they are reactive with the antigen-presenting molecule CD1d[2]. The homologous NKT-cell population in humans expresses an invariant TCR Vα24–Jα18 rearrangement; these TCR genes are orthologous to mouse *Vα14–Jα18* and the specificity of these cells for CD1d is highly conserved[95]. As a consequence of their limited TCR diversity, we and others have termed these T cells invariant (*i*)NKT cells[3]. The distinct developmental pathway for *i*NKT cells in the thymus[96] establishes them as a separate T-cell sublineage. *i*NKT cells can be found nearly everywhere conventional T cells are found, but with the exception of the mouse liver they are only a minority T-cell population, and they are even less frequent in humans. In mice, Vα14*i* NKT cells are either $CD4^+$ or double negative for CD4 and CD8, whereas a third population of $CD8^+$ Vα24*i* NKT cells exists in humans. On activation, *i*NKT cells exhibit cytotoxic activity, and they rapidly produce large amounts of cytokines, including interferon-γ and interleukin-4, conferring on them the ability to regulate immunity.

The term 'NKT cell' is ambiguous, however, because there are some cells that have the invariant TCR which do not express NK-cell receptors, and some conventional T cells can acquire NK-cell-receptor expression. Nowadays the more generic term 'NKT cell' is still widely accepted, but this can confuse even immunologists; a better nomenclature, based on the particular invariant TCR α-chain gene rearrangement, provides a more precise definition.

SH2D1A gene locus on the X chromosome, lack Vα24*i* NKT cells, and die from uncontrolled Epstein–Barr virus infections[56,57]. Similar findings have recently been reported for individuals defective for the X-linked inhibitor of apoptosis protein (XIAP)[58]. However, the data linking Vα24*i* NKT cells to protection from viral infections in humans is still fragmentary, and SAP-deficient individuals have a number of immune defects in addition to Vα24*i* NKT-cell deficiency.

Detrimental role for iNKT cells. *i*NKT cells are not always protective for the host, as illustrated by studies of chlamydia infection[59]. Following intranasal infection with *Chlamydia trachomatis* mouse pneumonitis (*C. muridarum*), *Cd1d*$^{-/-}$ mice showed less body-weight loss and decreased bacterial numbers in the lung compared with control mice. Furthermore, *Cd1d*$^{-/-}$ mice showed decreased production of interleukin (IL)-4 and IL-5, and lower levels of the immunoglobulin isotypes IgE and IgG1. These are the hallmarks of what immunologists call a T helper 2 (T_H2) response (BOX 2). T_H2 cytokines such as IL-4 can inhibit the other main type of T_H-cell response, the T_H1 response, which is important for microbial clearance because T_H1 immunity is characterized by the production of cytokines that activate macrophages. These results indicate that *i*NKT cells enhanced chlamydial infection through the augmentation of the T_H2 response, although for reasons that are not known, in other infections *i*NKT cells can promote a T_H1 response and microbial clearance. There are also data indicating that Vα14*i* NKT cells are detrimental in the cases of *Toxoplasma gondii*[60,61] and *Listeria monocytogenes* infection[62], but other laboratories have obtained different results[63–65] (TABLES 1,2), perhaps reflecting microbial strain differences or differences in complex experimental systems.

In summary, despite being a minority T-cell population, there is abundant evidence that *i*NKT cells are important in mice for host defence against various bacteria, viruses and parasites. There are also data, especially for viral infections, that indicate that this population could be important in humans as well. Work carried out in the last few years, described below, has begun to elucidate the two main mechanisms whereby *i*NKT cells help to protect the host from infections.

Indirect *i*NKT-cell activation

Cytokines and endogenous antigens can mediate iNKT-cell activation. How do *i*NKT cells, with such limited TCR diversity, respond to so many different infectious agents? Recent studies have shed light on the mechanisms by which *i*NKT cells are activated during infections (FIG. 4), including cases in which a microbial antigen for the invariant TCR is not present. It was reported that human Vα24*i* NKT cells produced IFN-γ in response to *Salmonella typhimurium* when cultured with DCs[66]. *S. typhimurium* lipopolysaccharide (LPS) or recombinant IL-12 could induce IFN-γ production by *i*NKT-cell clones in DC co-cultures, and IFN-γ production was inhibited by anti-IL-12 antibodies. Mouse DCs that are deficient for the adaptor myeloid differentiation primary-response gene 88 (MyD88), which interferes with much of the signalling by Toll-like receptors (TLRs), failed to induce IFN-γ. These data and the results from *in vivo* experiments indicate that TLR engagement, mediated in this case by *Salmonella* spp. infection or by LPS, induces IL-12 synthesis that is crucial for the activation of *i*NKT cells. This IL-12 was necessary but not sufficient, as the response could also be blocked with anti-CD1d antibodies[66].

More recently, it was shown that DCs from *Cd1d*$^{-/-}$ mice could not induce IFN-γ production by Vα14*i* NKT cells in response to *S. typhimurium*[67]. Neither could mice deficient for β-hexosaminidase (HEXB), an enzyme that is required for the synthesis of isoglobotrihexosylceramide (iGb3)[68], the first candidate endogenous antigen. Because *S. typhimurium* does not contain lipid antigens for the *i*NKT-cell TCR, these data indicate that *i*NKT cells were activated in this system by the combination of IL-12, induced by LPS engagement of TLRs expressed by DCs, and the recognition of relatively weak self ligands, such as iGb3, presented by CD1d (FIG. 4a). In this response, TCR recognition of a foreign microbial antigen is not involved, and therefore we refer to this pathway as an 'indirect mechanism' for *i*NKT-cell activation (FIG. 4a–c).

Bacterial infections can alter CD1D expression. Although the recognition of endogenous antigens is required for *i*NKT-cell activation in response to *S. typhimurium*, does bacterial infection increase *CD1D* expression and/or the presentation of stimulatory endogenous glycolipid ligands? Increased synthesis of the autologous GSL antigens including sulphatide, presented by CD1a or CD1b, and the ganglioside GM1, presented by CD1b, has been shown following the stimulation of monocytes with bacteria or bacterial components[69]. Regarding the CD1d isoform, there are several reports of increased

Toll-like receptor
(TLR). TLRs constitute a family of receptors of the innate immune system. Different members are found on the plasma membrane or on intracellular membranes. These molecules have leucine-rich repeats and they signal through adaptor proteins such as myeloid differentiation primary-response gene 88 (MyD88) to activate production of interleukin (IL)-12 and other cytokines. They are pattern-recognition receptors, meaning that they recognize certain molecules such as peptidoglycans, lipopolysaccharides or double-stranded RNA molecules that are broadly shared by many microorganisms.

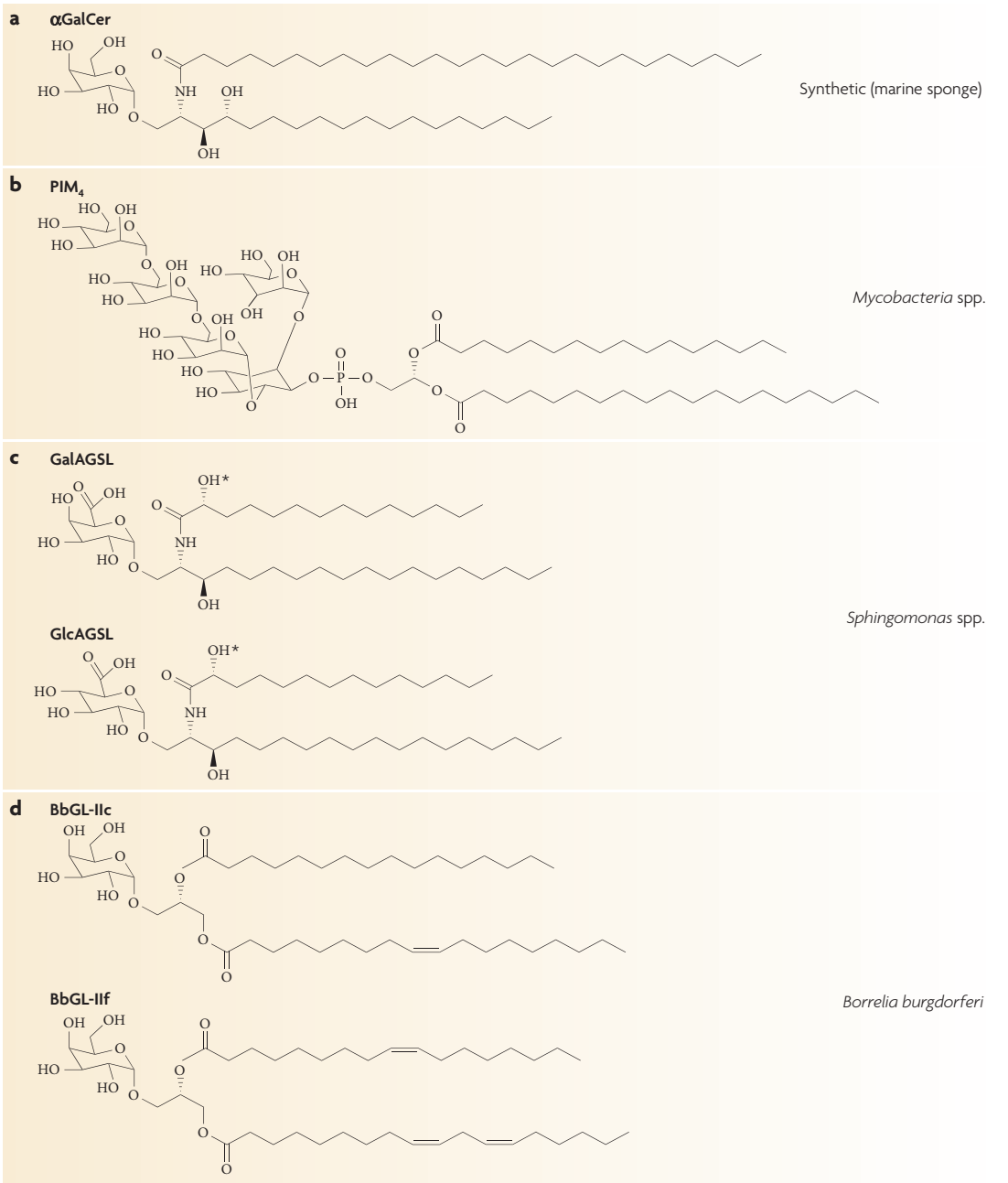

Figure 3 | **Structure of some microbial glycolipid antigens recognized by iNKT cells. a** | Structure of α-galactosyl ceramide (α-GalCer), the first known antigen for invariant natural killer T (iNKT) cells, originally extracted from a marine sponge. **b** | Structure of phosphatidylinositol tetramannoside (PIM_4), a weak iNKT-cell antigen originally extracted from *Mycobacteria* spp. **c** | Structure of GalAGSL (glycosphingolipid containing galacturonic acid) and GlcAGSL (glycosphingolipid containing glucuronic acid) originally extracted from *Sphingomonas* spp. The only difference between the two compounds being the noted 4′ hydroxyl on the sugar in the equatorial rather than the axial position. The asterisk indicates that the 2-hydroxyl on the acyl chain of the *Sphingomonas* spp. glycolipids is not always present. **d** | Structure of BbGL-IIc and BbGL-IIf, monogalactosyl diacylglycerol lipids, originally extracted from *Borrelia burgdorferi*. BbGL-IIc is the most potent BbGL-II antigen in a mouse model, whereas BbGL-IIf is the most potent in humans.

CD1D expression following exposure to bacteria or bacterial products. *CD1D* expression by DCs was increased when cultured *in vitro* with *S. typhimurium* or LPS from *Escherichia coli*[70], although the increased expression of CD1d on DCs was not observed *in vivo* during oral infection with *S. typhimurium*.

The upregulation of CD1d was also observed on macrophages transferred into *Mycobacterium tuberculosis*-infected mice[71]. When mouse macrophages were cultured with *M. tuberculosis* bacteria, bacterial lipids or the synthetic TLR2 agonist Pam_3Cys (tripalmitoyl-*S*-glyceryl cysteine), *Cd1d* expression was increased, but only if

Table 1 | **The role of Vα14*i* NKT cells in bacterial infection**

Microorganism and strain	Route of infection	Mouse strain	Disease in NKT-cell-deficient mice			Refs
			Cd1d$^{-/-}$	*Jα18*$^{-/-}$	Ab treatment	
Pseudomonas aeruginosa						
D4	Intranasal	B6 or BALB/c	Exacerbated	–	Exacerbated	38
PAO1	Intratracheal	B6	–	Not exacerbated	–	39
Sphingomonas spp.						
S. yanoikuyae	Intraperitoneal	B6	Exacerbated	Exacerbated	–	83
S. capsulata	Intravenous	B6	Exacerbated	Exacerbated	–	67
Mycobacterium tuberculosis						
Erdman	Intravenous	B6 or BALB/c	Not exacerbated	–	–	9,10
Kurono	Aerosol	BALB/c	–	Not exacerbated	–	11
H37Rv	Intravenous	B6	–	–	Exacerbated	12
Mycobacterium bovis BCG						
Pasteur	Intravenous	B6 or BALB/c	–	CFU→, inflammation↑	–	13
Tokyo 172	Intratracheal	B6	–	Not exacerbated	–	14
Salmonella spp.						
S. choleraesuis	Intraperitoneal	B6	–	CFU→, liver injury↓	–	15
S. typhimurium	Oral	B6	Not exacerbated	–	–	70
Listeria monocytogenes						
Strain unkown	Intravenous	B6	–	–	Ameliorated	62
10403S	Intravenous or oral	B6/129	Exacerbated	–	–	64
LO28	Oral	B6	–	Not exacerbated	–	65
Chlamydia spp.						
C. muridarum	Intranasal	BALB/c	Ameliorated	–	–	16,59
C. pneumoniae	Intranasal	B6 or BALB/c	Exacerbated	Exacerbated	–	16
Other						
Borrelia burgdorferi N40	Intradermal	B6 or B6/129	Exacerbated	–	–	40
Ehrlichia muris	Intraperitoneal	B6	Exacerbated	–	–	67
S. pneumoniae serotype 3	Intratracheal	B6	–	Exacerbated	–	37

Upwards arrow indicates an increase, downwards arrow indicates a decrease, and a horizontal arrow indicates no change.
Ab, antibody; B6, C57BL/6 strain inbred mice; CFU, colony forming unit; NKT, natural killer T cell.

recombinant IFN-γ was also added[71]. Recently, it was reported that *L. monocytogenes* induces increased *Cd1d* expression on macrophages and DCs *in vitro* and *in vivo*, which could be inhibited by anti-IFN-β antibodies[72]. Macrophages with increased *Cd1d* expression following exposure to bacteria or bacterial products were more effective at stimulating CD1d-dependent cytokine release by *i*NKT cells, even in the absence of exogenous antigen. These data indicate that antigen-presenting cells (APCs) stimulated with bacteria or TLR agonists can induce the activation of *i*NKT cells through the increased presentation of endogenous antigens by CD1d combined with IL-12 from activated DCs.

Endogenous antigen-mediated iNKT-cell activation. Another mechanism of indirect *i*NKT-cell activation was shown in a study of the response to *Schistosoma mansoni*. *i*NKT cells were activated during *S. mansoni* infection and had an important role in the augmentation of the T_H2 response[73]. When liver mononuclear cells (LMNCs) were cultured with *S. mansoni*-egg-sensitized DCs, the production of IFN-γ and IL-4 was significantly higher in cells from wild-type mice compared with cells from *Jα18*$^{-/-}$ mice or *Cd1d*$^{-/-}$ mice. *S. mansoni* eggs do not contain glycolipid antigens for the *i*NKT-cell TCR, and DCs from *Hexb*$^{-/-}$ mice, unable to synthesize the endogenous antigen iGb3, failed to induce IFN-γ and IL-4 production by LMNCs[74]. These

Table 2 | **The role of Vα14*i* NKT cells in parasite, fungal or viral infection**

Infective agent	Route of infection	Mouse strain	Disease in NK-cell-deficient mice			Refs
			Cd1d$^{-/-}$	*Jα18*$^{-/-}$	Ab treatment	
Parasite						
L. major	s.c. or i.v.	B6	Exacerbated	Exacerbated	Exacerbated	43,44
L. donovani	i.v.	BALB/c	Exacerbated	–	–	42
T. cruzi CL	i.p.	B6	Exacerbated	Exacerbated	–	45
T. cruzi Y or Tulahuen	i.p.	B6 or BALB/c	Not exacerbated	–	–	17,18
T. gondii Beverley	i.p.	BALB/c	–	–	Ameliorated	60
T. gondii 76K	Oral	B6	Not exacerbated	Ameliorated	–	61
T. gondii ME49	Oral	B6 or BALB/c	Exacerbated (B6)	Exacerbated (B6)	–	63
Schistosoma mansoni	Cutaneous	BALB/c	Worm→, Th2↓	–	–	73
P. berghei ANKA erythrocytes	i.p.	BALB/c	Exacerbated	–	–	19
Plasmodium spp.	i.p.	B6	Ameliorated	Ameliorated	–	
P. yoelii erythrocytes	i.p.	B6	Exacerbated	–	–	20
Fungal						
Cryptococcus neoformans	i.v. or i.t.	B6	–	Exacerbated (i.t.)	–	21,22
Viral						
HSV-1, SC16	Zosteriform	B6	Exacerbated	Exacerbated	–	46
HSV-1, KOS	Zosteriform or footpad	B6	Not exacerbated	–	–	47
HSV-2, 333	Intravaginal	B6	Exacerbated	–	–	48
Respiratory syncytial virus	Intranasal	B6	Virus↑, illness↑	–	–	23
		B6/129	Virus↑, illness↓			
		BALB/c	Virus→, illness↓			
Diabetogenic encephalomyo-carditis virus	i.p.	BALB/c	Exacerbated	Not exacerbated	–	24
Murine cytomegalovirus	i.p.	B6	–	Not exacerbated	–	25
Lymphocytic choriomeningitis virus	i.p. or i.v.	B6	Virus→, cytokine↑	–	–	26,27

Upwards arrow indicates an increase, downwards arrow indicates a decrease, and a horizontal arrow indicates no change; Ab, antibody; B6, C57BL/6 strain inbred mice; CFU, colony forming unit; HSV, herpes simplex virus; i.p., intraperitoneal; i.t., intratracheal; i.v., intravenous; NKT, natural killer T; s.c., subcutaneous.

data indicate that *i*NKT-cell activation by *S. mansoni*-egg-sensitized DCs was mediated by the recognition of iGb3. However, it remains controversial whether iGb3 is in fact the sole or crucial endogenous ligand for *i*NKT cells. Indeed, that DCs from *Hexb*$^{-/-}$ mice are inhibited in their stimulation of *i*NKT cells could be due to disruption of the endolysosomal vesicles where CD1d is loaded with antigen, as opposed to a deficiency in iGb3 synthesis[75].

Surprisingly, DCs from *Il12*$^{-/-}$ mice or *MyD88*$^{-/-}$ mice could induce cytokine production by LMNCs in a similar manner to wild-type DCs. These data suggest that, in response to *S. mansoni* egg antigens, *i*NKT cells can be activated by self glycolipids presented by CD1d, even in the absence of TLR signalling and IL-12 (FIG. 4b). It has not yet been determined, however, if *S. mansoni* egg extract increases the synthesis of endogenous glycolipid antigens and/or *Cd1d* expression. Similarly, it is not known if *S. mansoni*-egg-sensitized DCs are activated in a TLR-independent manner to produce innate immune cytokines, other than IL-12, that might contribute to the activation of *i*NKT cells.

***Cytokine-mediated* iNKT-cell activation.** It has recently been shown that Vα14*i* NKT cells also can be activated by IL-12 and IL-18 produced by DCs that have been activated by *E. coli* LPS, even in the absence of TCR stimulation by endogenous antigens presented by CD1d[76]. This purely cytokine-driven response constitutes a third type of indirect *i*NKT-cell activation. When mice were injected with *E. coli* LPS, Vα14*i* NKT cells produced IFN-γ but not IL-4, consistent with an inflammatory response that might enable *i*NKT cells to contribute to the induction of T_H1-type responses. Purified Vα14*i* NKT cells produced IFN-γ in response to *E. coli* LPS when cultured with wild-type DCs, but not with *Il12*$^{-/-}$ or *Il18*$^{-/-}$ DCs. Surprisingly, *Cd1d*$^{-/-}$ DCs were also able to induce IFN-γ production by Vα14*i* NKT cells. Furthermore, physiological concentrations of IL-12 and IL-18 together could induce IFN-γ production by purified Vα14*i* NKT cells, even in the absence of DCs. These data indicate that inflammatory cytokines such as IL-12 and IL-18, which are produced by TLR-stimulated DCs and other APCs, are necessary and sufficient for the induction of Vα14*i* NKT-cell activation (FIG. 4c). Finally, a recent paper indicates a fourth indirect activation pathway, in which the cytokine and the TCR signals needed for *i*NKT-cell activation are delivered by two different DC subsets[77].

We conclude that *i*NKT cells can be activated by infections indirectly through the activation of APCs even in the absence of microbial glycolipid antigens. By using any one of the indirect mechanisms described above, *i*NKT cells can sense the presence of many types of microorganism, including viruses that do not encode unique glycolipid antigens. This allows *i*NKT cells to make a rapid response to a wide range of infectious agents, similar to other cells that participate in the innate immune response. It is likely that a relatively weak TCR signal, delivered by self-glycolipid ligands presented by CD1d, can contribute to the signal delivered by innate-immune-cell cytokines. Furthermore, under some circumstances, such as those observed with exposure to *Schistosome* spp. egg extracts, the TCR signal in reaction to self glycolipids presented by CD1d might be sufficiently potent to mediate *i*NKT-cell activation. Although the indirect pathways are probably important, they do not account for the specificity and conservation of the invariant TCR.

Direct activation and bacterial antigens

Phospholipid antigens. Several groups have searched for microbial glycolipid antigens that directly activate the *i*NKT cells by engaging their invariant TCR. The first antigen reported was the glycophosphatidylinositol (GPI) anchor of proteins from *Plasmodium* spp. and *Trypanosoma* spp. It was asserted that the IgG responses to GPI-anchored surface antigens of *Plasmodium* spp. and *Trypanosoma* spp. were regulated through CD1d-restricted recognition of the GPI moiety by IL-4-producing NKT cells[78]. However, those findings remain controversial, as two subsequent studies did not reproduce the results[79,80].

Amprey *et al.* later showed that a subset of liver Vα14*i* NKT cells could be activated by a lipophosphoglycan (LPG) extract from *L. donovani*[42]. *L. donovani* is part of the genus *Leishmania*, the members of which are responsible for a wide array of diseases, ranging from self-healing cutaneous lesions to destructive skin and mucosal disease, and ultimately to fatal visceral infection. The authors observed an increase in parasite burden and a defect in the granulomatous response in *L. donovani*-infected mice deficient in Vα14*i* NKT cells. They also showed that a small percentage (3–6%) of the *i*NKT cells in the liver produced IFN-γ early after visceral *L. donovani* infection. They were able to show that this IFN-γ production by *i*NKT cells was IL-12 independent but CD1d dependent, indicating direct microbial antigen recognition by the *i*NKT-cell TCR instead of indirect recognition. *L. donovani* possesses a dense surface glycocalyx formed mainly by related glycoinositol phospholipids (GIPLs) and LPG. In a competitive assay, both purified GIPLs and LPG could inhibit α-GalCer-induced IL-2 production by Vα14*i* NKT-cell hybridomas, indicating that GIPLs and LPG can bind to CD1d and be potential microbial antigens. However, LPG–CD1d tetramer staining of liver mononuclear cells could not be achieved, and injection of purified LPG could stimulate only 1.4% of the liver NKT cells *in vivo*. Therefore, these data indicate that LPG could activate only a subset of Vα14*i* NKT cells.

A third early report on a potential microbial antigen for *i*NKT cells showed that several mammalian lipids, namely phosphatidylglycerol (PG) and phosphatidylinositol (PI), could specifically bind to CD1d[81]. Moreover, PI, but not PG, could induce IFN-γ production by splenic T cells from Vα14-transgenic mice. Those results

Glycocalyx
The general term referring to an extracellular network of polysaccharides that project from the cellular surfaces of some bacteria, epithelia and other cells.

Box 2 | **New math: T_H1 plus T_H2 is T_H0**

T cells orchestrate immunity largely through the production of cytokines that influence the behaviour of other cell types. The expression of groups of cytokine genes is coordinated, and originally two main patterns were recognized[97]. The T helper 1 (T_H1) pattern includes the production of interferon-γ (IFN-γ) and tumour-necrosis factor (TNF), cytokines that are important for activating macrophages and clearing intracellular pathogens[98,99]. The T_H2 pattern includes the production of interleukin (IL)-4, IL-5 and IL-13, cytokines that are important for the expulsion of helminth parasites. These cytokines cross-regulate one another. The T_H2 cytokine IL-4 prevents T_H1 immune responses and enhances T_H2 immune responses, whereas IFN-γ and other T_H1 immune-response-inducing cytokines, such as IL-12, inhibit T_H2 immune responses. The T-cell cytokine expression patterns are induced only after activation and cell division. Naive T cells (that is, those that have not encountered an antigen) produce lower levels of cytokines, mainly IL-2. Immunologists have subsequently recognized several other cytokine gene-expression patterns by differentiated T cells, including the recently described T_H17 pattern (characterized by IL-17-producing T_H cells) that might also have a role in microbial clearance through the activation of neutrophils[100]. Some T cells do not fit into one of the well-defined patterns, and the T_H0 pattern refers to cells that make mixtures of cytokines, including IL-4 and IFN-γ. *i*NKT cells are T_H0-like, as they acquire the ability to rapidly produce IL-4, IFN-γ and other cytokines, even as they differentiate in the thymus[2]. Despite this T_H0-like cytokine pattern, under some circumstances, *i*NKT cells can push the immune response in the T_H1 direction, whereas under other circumstances they direct T_H2 immune responses[1]. The mechanisms by which *i*NKT cells influence the immune response in these different directions remain to be defined.

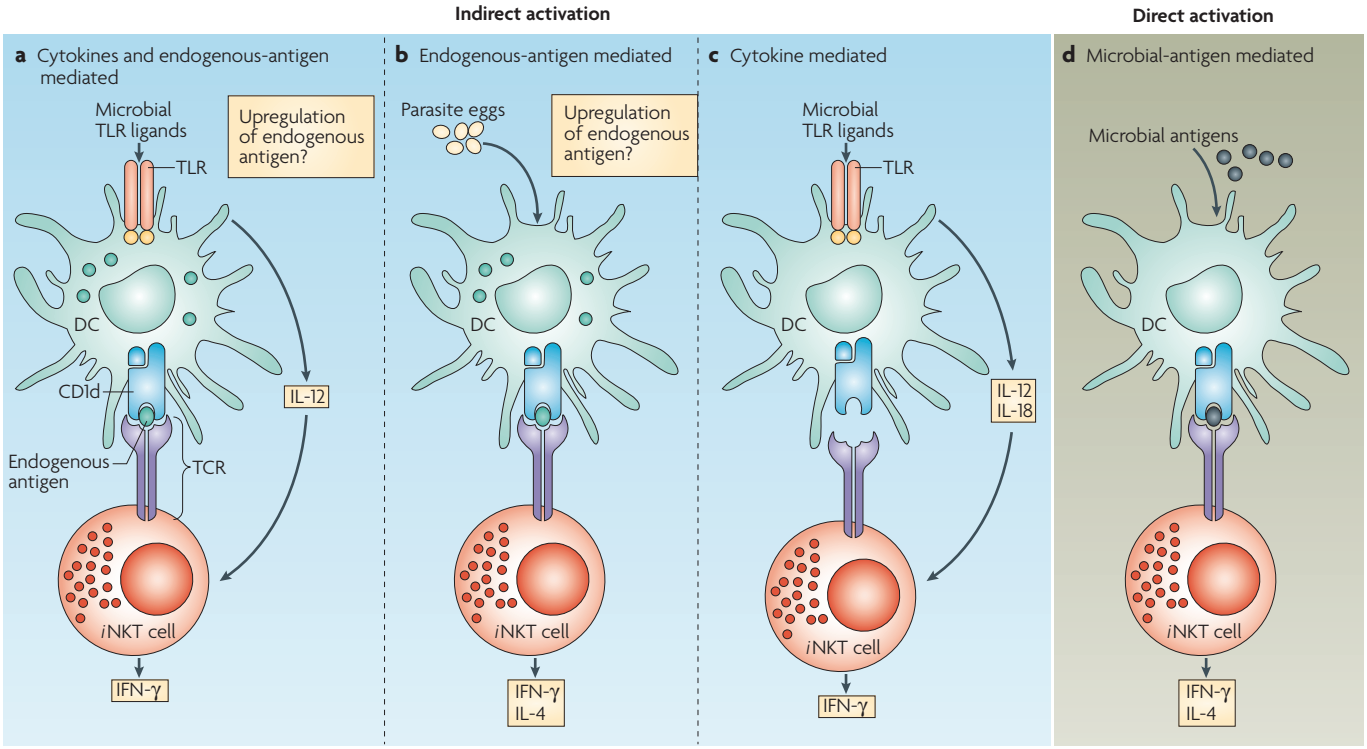

Figure 4 | **Invariant natural killer T (iNKT) cells have different pathways leading to their activation. a–c** | Indirect activation. The three indirect pathways do not depend on recognition by the *i*NKT-cell T-cell receptor (TCR) of a microbial antigen, but depend on cytokine release by activated dendritic cells (DCs) and/or the recognition of endogenous glycolipid ligands. **a** | Cytokine- and endogenous-antigen-mediated activation. During *Salmonella typhimurium* infection, lipopolysaccharide (LPS) stimulates Toll-like receptors (TLRs) on DCs and induces interleukin (IL)-12 release. *i*NKT cells are activated by the combination of IL-12 produced by LPS-stimulated DCs and recognition of endogenous antigen presented by CD1d. It has not been determined if LPS induces upregulation of endogenous antigen in DCs. **b** | Endogenous-antigen-mediated activation. *Schistosoma mansoni* egg-sensitized DCs induce IFN-γ and IL-4 production by *i*NKT cells. In this response, TLR-mediated activation of DCs is not involved. However, recognition of endogenous antigen is required. It has not been determined if endogenous antigen is upregulated in *S. mansoni* egg-sensitized DCs. **c** | *Escherichia coli*-LPS-stimulated DCs for IL-12 and IL-18 release. These cytokines are sufficient for IFN-γ production by *i*NKT cells, and recognition of endogenous antigen presented by CD1d is not necessary for *i*NKT-cell activation. **d** | Microbial-antigen-mediated direct activation. Glycosphingolipids from *Sphingomonas* spp. and galactosyl-diacylglycerols from *Borrelia burgdorferi* induce *i*NKT-cell activation by engaging their invariant TCRs. TLR-mediated DC activation, inflammatory cytokines such as IL-12 or recognition of endogenous antigen are not involved in this response. Modified with permission from REF. 102 © (2007) Macmillan Publishers Ltd.

prompted the authors to see if the same were true for a mycobacterial variant of PI. They found that a subset of mouse Vα14*i* NKT cells and human Vα24*i* NKT cells reacted to a variant of PI, a purified glycolipid extracted from a mycobacterium cell-wall fraction and enriched for PI tetramannosides (PIM_4) (FIG. 3b). Analysis with PIM_4–CD1d tetramers showed that the reactive cells comprised only a minority of the α-GalCer–CD1d tetramer-positive cells, especially in the mouse liver, indicating that PIM_4 reactivity, similar to the *Leishmania* spp. LPG antigen, might be dependent on special features of the more variable β-chain of the semi-invariant TCR, as the α-chains are identical. Another limitation of this study was that only purified glycolipid extracted from mycobacteria was used, leaving open the possibility that a minor constituent of the purified product was responsible for the Vα14*i* NKT-cell activation. Indeed, a later article reported that a synthetic version of PIM_4 did not stimulate *i*NKT cells, either *in vitro* or *in vivo*[82].

Natural glycosphingolipid antigens from* Sphingomonas *spp. The studies on *Leishmania* spp.- and mycobacteria-derived phospholipids helped establish the principle that the invariant TCR of *i*NKT cells recognizes glycolipids from microbial agents. However, because only a minority of the cells reacted, these specificities could not account for the conservation and selection of the invariant TCR. In 2005, the publication of three articles on GSLs from *Sphingomonas* spp. described antigens that are capable of activating essentially all *i*NKT cells[67,83,84]. *Sphingomonas* spp. are Gram-negative bacteria that lack LPS and are highly abundant in the environment, including soil, sea water and plants[85]. Prior to these studies, analyses of *Sphingomonas* spp. bacteria had revealed the presence of glycosylceramides in the cell wall, with structures similar to α-GalCer, including the rather unusual α-linkage of the sugar to the sphingosine-containing lipid[86,87]. The structures of two of these are shown in FIG. 3c. The abundance of these bacteria in

the oceans indicates that they could have been in the original marine-sponge sample that was used to identify α-GalCer, and their structural similarity to this potent antigen made them good candidates for antigens able to activate the population of *i*NKT cells.

By using purified or synthetic versions of these GSLs in several different *in vitro* assays, it was shown that a galacturonic-acid-containing GSL (GalAGSL), and one containing glucuronic acid (GlcAGSL)[83] (FIG. 3c) and similar compounds[67,84] could bind to CD1d. Moreover, although not as potent as α-GalCer, the *Sphingomonas* spp. GSLs could specifically activate mouse Vα14*i* NKT cells as well as human Vα24*i* NKT cells[67,83]. *Hexb*$^{-/-}$ DCs also could stimulate Vα14*i* NKT cells in response to *Sphingomonas* spp. These data indicate that the recognition of the putative self ligand iGb3 is not required for *i*NKT-cell activation in response to *Sphingomonas* spp.[67] Furthermore, TLR signalling and IL-12 were not required for GSL-mediated *i*NKT-cell activation, indicating that the observed activation was due to direct recognition of the microbial antigen by the TCR as opposed to the indirect pathway[67,83]. Direct recognition by the invariant TCR was confirmed by staining with CD1d tetramers loaded with the *Sphingomonas* spp. GSL. Indeed, in contrast to the LPG and PIM_4 studies, CD1d tetramers loaded with synthetic GalAGSL were able to detect at least 50% of the Vα14*i* NKT cells, defined as cells reacting with α-GalCer–CD1d tetramers. Moreover, the reactive cells were absent in *Cd1d*$^{-/-}$ and *Jα18*$^{-/-}$ mice, showing overlap between the α-GalCer-reactive and *Sphingomonas*-spp.-GSL-reactive populations[83]. *Ehrlichia* spp., which also belong to the class of α-proteobacteria, can activate *i*NKT cells independently of TLR signalling and iGb3 synthesis as well; this indicates the direct recognition of microbial antigen, although this antigen has not yet been defined[67].

Mice deficient for Vα14*i* NKT cells had a reduced clearance of *Sphingomonas* spp. bacteria at days 1–3 following infection[67,83]. The effect of Vα14*i* NKT cells on clearance was most evident in the liver[83], where these cells are most prevalent, but delayed clearance was also evident in the lung[67]; eventually, however, the bacteria could be cleared even in mice lacking Vα14*i* NKT cells. Therefore, direct recognition of GSLs by *i*NKT cells can contribute to the early stages of host defence against LPS-negative microorganisms. However, when a high dose of *Sphingomonas* spp. bacteria were administered, mice with *i*NKT cells died from shock whereas *i*NKT-cell-deficient mice were protected. This result provides another example in which an overactive protective *i*NKT-cell response is detrimental. The α-linked GSLs are believed to be unique to *Sphingomonas* spp. and related bacteria, but these bacteria are not highly virulent or pathogenic in humans, although infections in immunocompromised patients have been reported[88,89]. It remained uncertain whether *i*NKT cells can recognize other classes of glycolipids that might be derived from pathogenic microorganisms.

***Galactosyl diacylglycerol antigens from* Borrelia burgdorferi.** *B. burgdorferi* has recently been shown to have a different category of glycolipid antigen that activates the TCR of *i*NKT cells[79]. These spirochetes lack LPS but, contrary to *Sphingomonas* spp., they are pathogenic. *B. burgdorferi* is the causative agent of Lyme disease, which, with 15,000 cases each year, is the most common vector-borne disease in the USA[90]. *Cd1d*$^{-/-}$ mice infected with *B. burgdorferi* have been reported to have an increased bacterial burden, and they develop increased thickening of the tibiotarsal joint, indicative of arthritis[40]. During *B. burgdorferi* infection, either after injection of live spirochetes or by using infected ticks, Vα14*i* NKT cells were activated, as seen by an increase in both CD25 and CD69 expression on α-GalCer–CD1d tetramer-positive cells[79]. Similar data were obtained when DCs pulsed with bacterial lysates were injected into the mice. *B. burgdorferi* expresses two abundant glycolipids (*B. burgdorferi* glycolipid (BbGL)-I and BbGL-II)[91]. BbGL-I is a cholesteryl 6-O-acyl-β-galactoside (structure not shown) and BbGL-II is a 1,2-diacyl-3-O-α-galactosyl-*sn*-glycerol (FIG. 3d). BbGL-II, but not BbGL-I, was able to induce IL-2 secretion by Vα14*i* NKT-cell hybridomas, although it was not as potent as α-GalCer or the *Sphingomonas* spp.-derived GalAGSL. The BbGL-II isolated from the bacteria contained a mixture of C14:0, C16:0, C18:0, C18:1 and C18:2 fatty acids, with C16:0 and C18:1 being the most abundant, and it was uncertain which fatty acid(s) was linked to the *sn1* position of the glycerol and which was linked to the *sn2* position. Therefore, to further define *i*NKT-cell specificity, eight chemically synthesized variants of BbGL-II were tested in several assays. BbGL-IIc, which consists of an oleic acid in the *sn1* position and a palmitic acid in the *sn2* position (FIG. 3d), was by far the most potent antigen[82].

By using MyD88-deficient mice or TRIF-deficient *Trif*$^{Lps2/Lps2}$ mice, the authors showed that BbGL-IIc induced *in vitro* proliferation, as well as *in vivo* activation of Vα14*i* NKT cells independently of the presence of MyD88 or TRIF, indicating that Vα14*i* NKT-cell activation induced by BbGL-IIc is not dependent on TLR signals and the indirect pathway, but rather is due to direct recognition of the antigen by the TCR of the Vα14*i* NKT cells. This broad reactivity by the TCRs expressed by *i*NKT cells was confirmed by tetramer staining. CD1d tetramers loaded with BbGL-IIc detected approximately 23% of the liver Vα14*i* NKT cells compared with α-GalCer-loaded tetramers. This might be an underestimate of the extent of reactivity to this relatively weak antigen in the Vα14*i* NKT-cell population, as BbGL-IIc was able to activate all of the Vα14*i* NKT-cell hybridomas tested. Therefore, a substantial fraction of the Vα14*i* NKT cells can probably directly recognize α-galactosyl diacylglycerols derived from *B. burgdorferi* by direct engagement of their invariant TCR.

The *i*NKT-cell response to *B. burgdorferi* glycolipids is conserved in humans. Vα24*i* human NKT cell lines produced IFN-γ and IL-4 after culture with cells transfected with CD1d in the presence of the synthetic galactosyl diacylglycerol compounds[82]. The response pattern was different from that observed with mouse Vα14 *i*NKT cells; minimal cytokine release was induced

by BbGL-IIc and maximal responses were observed after culture with compounds having a higher degree of unsaturation in the acyl chains, in particular BbGL-IIf (FIG. 3d).

Because BbGL-IIc is a diacylglycerol-based molecule and not a GSL, these data have several important implications for understanding the biology of *i*NKT-cell responses to infectious agents. First, because bacteria other than *B. burgdorferi* have glycoglycerol lipids, the invariant TCR could then have a broad reactivity to various microorganisms, which might in part explain the evolutionary selection for this TCR specificity. Second, by altering the degree of unsaturation of the acyl chains, we speculate that bacteria could evade recognition by *i*NKT cells by producing glycolipids that can bind to CD1d but do not activate the invariant TCR.

Conclusions and perspectives

The recent findings on *i*NKT cells show that they have important roles in host defence against various pathogens. Regarding the direct-recognition pathway, bacterial glycolipid antigens are recognized by the invariant TCR, including glycosphingolipids found in *Sphingomonas* spp., and glycerol-based antigens that have a wider distribution. It is certain, however, that some bacteria, such as *Salmonella* spp. and *E. coli*, do not have glycolipids for the invariant TCR. It therefore remains to be determined which types of microorganism have such antigens, and if this is confined predominantly to certain types — for example, extracellular bacteria, those that are not Gram-positive or those lacking LPS. Moreover, parasite antigens that unequivocally activate the invariant TCR have not been reported, although it is likely that such antigens exist in some species. It is also unknown if microbial modulation of the composition of the fatty-acid chains is an effective mechanism for the evasion of the *i*NKT-cell response, or if some bacteria inhibit CD1d expression, similar to viruses. Finally, whereas the conservation of the *i*NKT-cell specificity is striking, there is still relatively little evidence that human *i*NKT cells are important for microbial clearance, and additional studies are clearly needed. Moreover, some species such as cattle lack CD1d and *i*NKT cells. This might reflect exposure to different pathogens or the redundant functions of group I CD1 molecules.

Ultimately, however, if *i*NKT cells are important, it might be possible to incorporate glycolipids that activate *i*NKT cells into vaccines. Because CD1d molecules are not polymorphic, and *i*NKT cells respond similarly in different individuals, it should be possible to design 'one-size-fits-all' glycolipid agents.

The indirect activation pathway allows *i*NKT cells to respond to many infectious agents. Future studies will probably delineate the different mechanisms whereby *i*NKT cells are activated by viral as opposed to bacterial infections, the relevant APC types, the innate sensors, including TLRs and other innate sensors used to detect the different infections, and the cytokine and antigen-presentation pathways that communicate with the *i*NKT cell, leading to activation. A better definition of the relevant self antigens is also required.

In addition to host defence, *i*NKT cells have been implicated in several chronic inflammatory conditions, including asthma[92] and even atherosclerosis[93,94]. It will be important to determine if *i*NKT-cell activation in these contexts depends on direct activation mediated by specific microbial glycolipid antigens from commensal or pathogenic microorganisms, or alternatively, whether *i*NKT-cell activation depends predominantly on cytokines from innate immune cells.

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Competing interests statement
The authors declare no competing financial interests.

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