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Innate-like recognition of microbes by invariant natural killer T cells

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Abstract

Invariant natural killer T cells (*i*NKT cells) express a restricted T cell antigen receptor (TCR) repertoire and they respond rapidly to glycolipid antigens presented by CD1d. These glycolipid antigens have hexose sugars in α -linkage to two types of lipids that can bind to CD1d. Recent work has shown that the responses of *i*NKT cells to antigen-bearing microbes can have a profound impact on the development of inflammatory diseases. *i*NKT cells overcome the limitation of their limited TCR diversity by also responding in a foreign antigen-independent fashion to some infectious agents, similar to NK cells. Recent results demonstrate several mechanisms for the indirect activation of *i*NKT cells by viruses or TLR ligands, dependent on self-antigen recognition and/or different cytokines produced by antigen presenting cells. The means by which *i*NKT cells influence other cell types and overall host defense are likewise diverse, illustrating the flexibility and functional diversity of this T lymphocyte sublineage.

Introduction

Many mammals, including rodents and primates, have populations of invariant natural killer T cells (*i*NKT cells). These cells are *bona fide* T lymphocytes that express an $\alpha\beta$ T cell antigen receptor (TCR) and that are subject to thymus selection, but their immune responses bear a striking resemblance to innate immune cells. Although *i*NKT cells are reported to have many functions in regulating immune responses, in part through the recognition of self-antigens presented by CD1d, it is our contention that a principal driving force behind the conservation of this population is the detection of microbial infections.

Salient properties of *i*NKT cells that distinguish them from conventional T lymphocytes include [1–3]: 1) the co-expression of $\alpha\beta$ T cell antigen receptors (TCRs) and receptors typically found on NK cells. 2) expression of a semi-invariant TCR, hence their designation as *i*NKT cells. This TCR is composed of an invariant TCR α chain, encoded by a V α 14-J α 18 rearrangement in mice (V α 14i NKT cells) and a homologous V α 24-J α 18 rearrangement

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Conflict of interest

None.

(V α 24i NKT cells) in humans. 3) recognition of glycolipid antigens presented by CD1d, a MHC class I-like antigen presenting molecule that is not highly polymorphic. The specificity of antigen recognition is highly conserved if mouse and human *i*NKT cells are compared. 4) innate-like immune responses characterized by the ability to produce cytokines such as IL-4 and IFN γ almost immediately after activation. Furthermore, there is no evidence for a memory-like long-term expansion of this population after antigenic stimulation.

We can classify the mechanisms of activation of *i*NKT cells by microbes into two categories that are summarized in Table 1. First, there is direct activation of the invariant TCR by microbial glycolipids presented by CD1d. Second, there are foreign antigen-independent or indirect mechanisms, which depend on the responses of antigen presenting cells (APC) to microbes. These APC responses include the production of cytokines such as IL-12, and/or the presentation of self-glycolipid antigens by CD1d. The indirect mechanisms allow *i*NKT cells to respond rapidly to diverse microbes despite a limited TCR diversity. Here we review recent findings on the responses of *i*NKT cells to microbial infections, with particular emphasis on the responses to bacteria and viruses.

Microbial glycolipids recognized by *i*NKT cells

The first antigen identified that is recognized by *i*NKT cells was α -galactosyl ceramide (α GalCer). This compound was derived from the marine sponge *Agelas mauritanicus* in a screen for a substance that could prevent the metastases of tumors to the mouse liver [4]. It was later shown to act by stimulating V α 14i NKT cells [5]. α GalCer is a glycosphingolipid (GSL), a type of glycolipid with ceramide as the lipid moiety (Figure 1), and which also includes the gangliosides found in many organisms, including mammals. What distinguishes α GalCer from other GSLs is the α -linkage of the 1' carbon of the galactose to the ceramide lipid, as most of the GSLs in nature have a β linkage. GSLs with the β linkage are not antigenic for *i*NKT cells.

In the literature, there are only two types of bacteria that have glycolipid antigens that can activate most *i*NKT cells, and where *i*NKT cells are required for optimal host protection or bacterial clearance. This story is rapidly evolving, however, as several additional examples of microbes having *i*NKT cell antigens were reported at a recent meeting [6].

Recognition of glycosphingolipids

The first example of a microbial glycolipid recognized by *i*NKT cells is provided by *Sphingomonas spp.*, which are gram-negative bacteria that lack LPS [7–9]. *Sphingomonas* have abundant GSLs with α -linked sugars, similar to α GalCer [10,11]. *Sphingomonas* are found in soil and seawater [12], and therefore it is likely that the α GalCer obtained in the original sponge isolate contained these bacteria as the source of the glycolipid antigen. *Sphingomonas* are unique in having GSLs in their outer membrane.

Although they are not highly pathogenic [13,14], recent work suggests a possible role for *Sphingomonas spp.* in the causation of primary biliary cirrhosis (PBC), in a mouse model of this disease [15•]. PBC is an autoimmune disease characterized by the presence of anti-mitochondrial antibodies (AMA) and autoimmune attack on the bile ducts [16]. A major target of the AMA is pyruvate dehydrogenase complex E2 (PDC-E2), and there is a striking degree of similarity comparing mammalian PDC-E2 to the sequence in *Novosphingobium aromaticivorans*, a member of the *Sphingomonas* genus [17,18]. Injection of *N. aromaticivorans* into mice caused the CD1d-dependent formation of PDC-E2 antibodies [15•]. The mice eventually also developed a CD1d-dependent inflammation of the liver with characteristics of PBC, including infiltration of the bile ducts, liver hypertrophy and granuloma formation. Although live bacteria were required for chronic disease, the long-term inflammation in the liver was resistant to antibiotic treatment, and the data suggest that

autoimmunity dependent on conventional T cells eventually develops in the face of sterile immunity [15•]. Therefore V α 14i NKT cells play a role in the initiating phases rather than in chronic disease. Extrapolating these results to humans, approximately 25% of PBC patients have detectable rDNA from *Sphingomonas* bacteria in their intestine, as do a similar proportion of the healthy controls [17]. This raises the question as to why only a few people develop PBC when many are exposed *N. aromaticivorans*.

A related issue is whether there is anything special about the antigen-driven activation of *i*NKT cells by *N. aromaticivorans* compared to other *Sphingomonas* species. Interestingly, a *Sphingomonas* bacterium can produce more than one type of GSL, and different species differ in their GSLs [10,11,19]. The GSLs can differ with regard to the length and structure of the sphingosine base, as well as the number of carbohydrates. For example, while *S. yanoikuyae* has GSLs with α -linked monosaccharides containing either galacturonic or glucuronic acid (Figure 1), *S. paucimobilis* and *S. adhaesive* also synthesize tetrasaccharide-containing GSLs with the α -linked glucuronic acid further elaborated with three additional sugars [10,11,19]. The oligosaccharide-containing GSLs are weak antigens or not antigenic at all, however, due in part to a failure to efficiently process the complex carbohydrate structures to the antigenic, monosaccharide form [20•,21•]. These data suggest that *Sphingomonas* spp. may differ in their ability to activate *i*NKT cells, based in part of the composition of their GSLs. *N. aromaticivorans* may or may not be exceptional with regard to its ability to activate *i*NKT cells. Regardless, it is possible that the biosynthesis of certain GSLs, which may compete for CD1d binding but that inhibit *i*NKT cell activation, may constitute a type of immune evasion mechanism.

Recognition of diacylglycerol glycolipids

The second example of a bacterium having a glycolipid antigen that activates *i*NKT cells in *Borrelia burgdorferi*. *B. burgdorferi* is a pathogen without qualification, as this spirochete is the cause of Lyme disease, the most common vector-borne disease in the United States [22]. Infection with *B. burgdorferi* results from the bite of *Ixodes scapularis* ticks, and if not treated promptly with antibiotics, a multisystem inflammatory disorder develops that targets the skin, joints, heart, and nervous system. Mice develop a similar chronic inflammation, with severity dependent on the inbred strain [23]. Interestingly, the glycolipid antigen from *B. burgdorferi* that activates *i*NKT cells has a diacylglycerol lipid (Figure 1) rather than a ceramide lipid [24]. What the *Sphingomonas* and *Borrelia* antigens have in common, however, is an α -linked hexose sugar, which in the case of *B. burgdorferi* is a galactose [7–9,24,25]. The glycosylated diacylglycerol antigen for *i*NKT cells from *B. burgdorferi*, *Borrelia burgdorferi* glycolipid II (BbGL-II), is abundant in the spirochete [26]. Mice lacking the J α 18 segment are deficient for V α 14i NKT cells because they cannot form the invariant α chain of the TCR [27]. These mice have reduced spirochete clearance and they are more susceptible to chronic inflammation following *B. burgdorferi* infection, although the outcome is highly dependent on the mouse strain background [28•,29•]. In BALB/c mice, the effect of V α 14i NKT cell deficiency was more evident in the joint than in the heart [28•]. Furthermore, there was not a local accumulation of V α 14i NKT cells in the joint tissue, suggesting the cells were acting systemically. By contrast, in C57BL/6 mice, the effect was more prevalent in the heart, and there was evidence for a local accumulation of V α 14i NKT cells and IFN γ in heart tissue [29•].

Indirect recognition of microbes

There are many examples of immune responses influenced by *i*NKT cells where a foreign antigen recognized by the invariant TCR has not been identified and is presumed not to exist [30]. This includes the responses to some bacteria, such as *Salmonella typhimurium* [31], but

the responses to viral infections perhaps provide the clearest example of *i*NKT cell activation in the absence of a foreign antigen. The response CD8⁺ T cell response to several viruses, such as influenza virus, can be augmented by α GalCer [30,32–35]. While this could be important for the development of glycolipid adjuvants, the cytokine storm induced by this highly potent antigen likely does not represent the normal, physiologic role of *i*NKT cells in anti-viral responses. In this article, we therefore emphasize those studies indicating a role for *i*NKT cells in the indirect recognition of viruses and other microbes that do not depend on pharmacologic activation of *i*NKT cells with synthetic glycolipids.

TLR ligands activate *i*NKT cells

The use of Toll-like receptor (TLR) ligands as a stimulus provides a model for the indirect activation of *i*NKT cells by infectious agents. Lipopolysaccharide (LPS), a ligand for TLR4 [8,31,36••,37••] and unmethylated CpG oligodeoxynucleotides (CpG ODN), which signal via TLR 9 [37••,38••,39•] activate *i*NKT cells in vitro and in vivo to produce IFN γ but not IL-4. These TLR ligands activate APC, which then stimulate *i*NKT cells. Several mechanisms have been reported (Table 1). For LPS, these include the secretion of IL-12 by APC combined with a CD1d-dependence that is strongly suggestive of self-antigen presentation [8,31]. LPS has been reported to alter the synthesis of GSLs in monocytic cell lines [40], which might be related to increased self-antigen presentation—if the self-antigen were in fact a GSL. Furthermore, LPS synergized with IFN γ to increase the level of surface CD1d expression by macrophages [41]. In another study, by contrast, LPS-mediated activation of V α 14*i* NKT cells required a combination of IL-12 and IL-18 secretion by APC, but not CD1d expression, and therefore presumably did not require antigen presentation by CD1d [36••]. Physiologic levels of IL-12 and IL-18 could activate V α 14*i* NKT cells in vivo to produce IFN γ and to alter their patrolling behavior in the liver so that they adhered to the liver sinusoidal endothelium [42•]. This cytokine driven response is similar to the cytokine-mediated activation of NK cells, highlighting the innate-like function of V α 14*i* NKT cells.

Similarly to the LPS response, several mechanisms have been reported for CpG ODN-mediated activation of V α 14*i* NKT cells. In one report, this depended on type I interferon secretion by APC, and the biosynthesis of a putative GSL self-antigen [37••]. In two subsequent studies, however, this activation event depended on TLR9 and MyD88 sensing of the CpG-ODN by APC, leading to IL-12 secretion, with CD1d expression and self-antigen presentation not playing a major role [38••,39•]. Several reports have documented the activation of V α 24*i* NKT cells by CpG ODN [43–44,45••]. This required type I interferon secretion by plasmacytoid DC (pDC) that in turn activated myeloid DC to stimulate V α 24*i* NKT cells. The different outcomes probably relate to differences in the source and dose of the TLR ligand used, as well as the different methods for preparing the APC used in the in vitro studies. Regardless, the divergent results emphasize the existence of multiple pathways that can lead to the indirect activation of *i*NKT cells.

Viral activation of V α 14*i* NKT cells

Recent studies have uncovered the mechanism for V α 14*i* NKT cell activation following infection with mouse cytomegalovirus (MCMV) a well-studied β herpes virus [38••,46,47••]. V α 14*i* NKT cells produced IFN γ but not IL-4 in vivo by 24–36h after MCMV infection, and this response required TLR9 expression by the APC, and the secretion of IL-12, with a partial dependence on type I interferon secretion. CD1d expression, however, was not required. Therefore, the pathway leading to the stimulation of V α 14*i* NKT cells by MCMV infection is most similar to the IL-12-dependent pathway described above [38••,47••]. The activation of V α 24*i* NKT cells by irradiated herpes simplex virus 1 (HSV1) also follows the mechanism outlined for activation by CpG ODN. It requires type I interferon secretion by human pDC,

which do not express CD1d, to activate myeloid DC to increase CD1d expression [45••]. Myeloid DC activated the $V\alpha 24i$ NKT cells in a fashion that is partially dependent on CD1d expression, implicating self-antigen recognition in this process.

The consequences of viral activation of i NKT cells for host defense vary depending on the virus and the location of the anti-viral response examined. For example, following infection with lymphocytic choriomeningitis virus (LCMV), $V\alpha 14i$ NKT cells are important for controlling viral replication in the pancreas and liver, but not in the spleen [48••]. The control of LCMV replication depended on the ability of $V\alpha 14i$ NKT cells to recruit pDC to the liver and pancreas, and to activate the pDC to increased secretion of type I interferon to inhibit viral replication. The $V\alpha 14i$ NKT cell-pDC interaction is dependent on OX40 expressed by $V\alpha 14i$ NKT cells interacting with OX40L expressed by pDC. The different outcome in the spleen was attributed to reduced OX40 expression by $V\alpha 14i$ NKT cells there.

The effects of $V\alpha 14i$ NKT cells following intranasal infection with influenza virus, however, are different. In the absence of $V\alpha 14i$ NKT cells, myeloid derived suppressor cells (MDSC) expand in the lung after infection. $V\alpha 14i$ NKT cells prevented this by a mechanism that depended on CD1d expression in the host and the presentation of self-antigen [49••]. It also requires CD40 expressed by the $V\alpha 14i$ NKT cells interacting with CD40L expressed by the MDSC. This interaction leads to a conversion of the myeloid cells from their suppressive function, with increased IL-12 secretion by these cells and decreased production of immune suppressive molecules such as arginase 1 and nitric oxide synthase 2

Conclusions and future perspectives

There is now abundant evidence that i NKT cells can be activated by diverse microbial infections, the limitation of their restricted TCR repertoire is overcome because they can react even in those cases in which a foreign antigen presented by CD1d is not produced. The two general mechanisms for i NKT cell activation are well established, a foreign antigen-dependent mechanism typical of adaptive immunity, and a group of mechanisms independent of foreign antigen that bear a resemblance to the responses of NK cells and other cells of the innate immune system. GSL antigens are found only in *Sphingomonas spp.*, but the discovery of glycosylated diacylglycerol antigens suggests that many other types of bacteria and even protozoan parasites could have glycolipid antigens for the invariant TCR. This is an emerging and rapidly moving area of research. Any infection with antigen-bearing bacteria is almost certain to activate i NKT cells through a combination of the direct and indirect pathways, and the interplay between these two activation pathways has yet to be explored. It must be noted, too, that formal proof that the glycolipid antigen-specific i NKT cell responses are in fact protective has not been obtained. This would require the generation of isogenic microbial strains having and lacking the ability to synthesize the glycolipid antigen in question, a significant challenge considering the abundance of these glycolipids and their likely importance for bacterial viability. Regarding the indirect pathway, identification of the relevant self-antigens clearly is a prerequisite for understanding how microbial infection and cytokines from innate immune cells interface with the synthesis and presentation of self-antigens. The frequency of $V\alpha 24i$ NKT cells varies greatly in the peripheral blood of humans. While genetic differences almost certainly play a role in this variability, the role of infection as a determinant of i NKT cell frequency and responsiveness requires further exploration. However, even at a frequency of 0.1% or less, it is plausible that the rapid and copious cytokine responses by i NKT cells could have a strong influence on host defense.

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Sphingomonas* spp.** Borrelia burgdorferi*** **Figure 1.**

Table 1

Summary of iNKT cell activation by microorganism

Type of activation	Stimuli	Microbial antigen	Endogenous antigen	Cytokine	TLR signaling	Ref
Direct	<i>Sphingomonas</i>	GSL	-	-	-	7-9
	<i>B. burgdorferi</i>	Diacylglycerol	-	-	-	24
	<i>S. typhimurium</i> LPS	-	+	IL-12	n.t	8,31
Indirect	<i>S. typhimurium</i> LPS	-	-	IL-12	n.t.	36**
	<i>E. coli</i> LPS	-	-	IL-12	n.t.	36**
	CpG	-	+	Type I IFN	n.t.	37**
	CpG	-	-	IL-12	TLR9	38**
	MCMV	-	-	Type I IFN	TLR9-	38**, 47**
	HSV1	-	+	Type I IFN	n.t.	45**

iNKT cells can be activated by TCR stimulation with microbial antigens (direct activation) or with endogenous antigens and/or cytokines (indirect activation). GSL; glycosphingolipid, DAG; diacylglycerol, +: required, -: not required or not involved. n.t.; not tested