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The role of invariant natural killer T cells in microbial immunity

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Abstract

Invariant natural killer T cells (iNKT cells) are unique lymphocytes with characteristic features, such as expression of an invariant T cell antigen receptor (TCR) α chain, recognition of glycolipid antigens presented by CD1d molecules, and ability to rapidly produce large amounts of cytokines, including interferon- γ (IFN γ) and interleukin 4 (IL-4) upon TCR stimulation. Many studies have demonstrated that iNKT cells participate in immune response against diverse microbes, including bacteria, fungi, protozoan parasites and viruses. Generally, these cells play protective roles in host defense against infections. However, in some contexts they play pathogenic roles, by inducing or augmenting inflammation. Recent reports show that iNKT cells recognize glycolipid antigens from pathogenic bacteria including *Streptococcus pneumoniae*, and they contribute to host defense against infection. iNKT cell responses to these microbial glycolipid antigens are highly conserved between rodents and humans, suggesting that iNKT cells are evolutionally conserved because their invariant TCR is useful in detecting certain pathogens. Furthermore, glycolipid-mediated iNKT cell activation during immunization has adjuvant activity, enhancing humoral and cell-mediated responses. Therefore, iNKT cell activation is an attractive target for developing new vaccines for infectious diseases.

Keywords

NKT cell; glycolipid; CD1d; invariant; bacteria

Introduction

Natural killer T (NKT) cells are innate-like lymphocytes that co-express both a T cell antigen receptor (TCR) and NK receptors [1–5]. In contrast to conventional T cells that recognize peptide antigens presented by major histocompatibility complex (MHC) class I and II molecules, NKT cells recognize glycolipid antigens presented by CD1d, an MHC class I-like antigen-presenting molecule. Crystal structure analyses show that the CD1d molecule possesses two antigen-binding grooves, called the A and F pockets [5, 6]. These pockets are deep, narrow and hydrophobic, making them suitable for binding the lipid tails of glycolipid antigens [5, 6].

A major subset of NKT cells expresses an invariant TCR that uses the V 14-J 18 gene segments in mice, and the homologous V 24-J 18 in humans. These cells are referred to

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Conflict of interest

None

type I [7] or as invariant NKT (α NKT) cells [1–5]. α -galactosylceramide (α -GalCer), a synthetic homologue of marine sponge-derived glycolipid, was the first α NKT cell antigen discovered, and this highly potent glycolipid antigen has been critical for understanding the functions of these cells [8]. Upon antigen recognition, α NKT cells rapidly produce large quantities of cytokines, including both interferon- γ (IFN γ) and interleukin 4 (IL-4), in addition to others, and they express costimulatory molecules such as CD40 ligand (CD40L) [1–4]. Activated α NKT cells can stimulate dendritic cells (DCs), NK cells and other immune cells through cytokines such as IFN γ , and expression of CD40L, leading to enhancement of the innate immune response, which ultimately contributes to augmenting acquired immunity. With these unique features, α NKT cells participate in mouse models of various immune and inflammatory responses including cancer immunity, microbial immunity, autoimmunity, atherosclerosis and asthma.

The α NKT cell response to glycolipid antigens is highly conserved between human and mouse. Mouse α NKT cells can recognize glycolipid antigens presented by human CD1d, while human α NKT cells can recognize the same antigen presented by mouse CD1d molecules [9]. This evolutionary conservation suggests that the α NKT cell response also is an important immune mechanism for humans. Therefore, findings obtained from mouse studies may provide useful information for understanding the possible roles of human α NKT cells in immune responses.

Another population of CD1d reactive NKT cells exists that expresses a more diverse TCR repertoire; these are sometimes called type II NKT cells. Sulfatide has been shown to activate some of these type II cells, and there is evidence they exert suppressive roles in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis [10]. In contrast to the protective role of α NKT cells in tumor immunity, sulfatide reactive NKT cells were shown to inhibit anti-tumor responses [11]. While new antigens for type II NKT cells are continually being discovered, the function of these cells in microbial immunity remains to be elucidated [12, 13].

In this review, we focus on α NKT cells and summarize the roles of these cells in response to diverse microbes concentrating on selected examples of infections by bacteria, fungi, protozoan parasites and viruses. We describe how intestinal microbes affect α NKT cell function and regulate inflammation. We also review recent discoveries of bacterial glycolipid antigens recognized by α NKT cells and their importance in microbial immunity.

Role of α NKT cells in host defense against infections

Bacterial infections

It has been shown that α NKT cells contribute towards host defense against a number of bacterial infections; a few examples are summarized here. *Streptococcus pneumoniae* is a leading cause of community acquired pneumonia and secondary bacterial pneumonia post influenza virus infection. Following pulmonary infection with *S. pneumoniae*, J β 28-deficient J β 28KO mice that lack α NKT cells [14] had significantly higher bacterial burden in the lung and a lower survival rate compared to wild-type (WT) mice [15]. Neutrophil recruitment to the lung was impaired in J β 28KO mice in accordance with lower production of neutrophil recruiting cytokines, including tumor necrosis factor (TNF), as well as reduced macrophage inflammatory protein 2 (MIP-2). Furthermore, transfer of liver mononuclear cells (20–40% α NKT cells) from WT mice into J β 28KO mice restored neutrophil accumulation through increased production of TNF and MIP-2, resulting in bacterial clearance [16]. However, transfer of liver mononuclear cells from J β 28KO or IFN γ KO mice into J β 28KO mice did not lead to recovery of neutrophil accumulation and a normal level of bacterial clearance,

suggesting that IFN γ , possibly from λ NKT cells, plays an important role in host defense against *S. pneumoniae* infection.

It should be noted that J λ 18KO mice have recently been reported to have deficits in rearrangements of approximately 60% of their J λ segments, all of those upstream from J λ 18, likely due to insertion of the gene encoding neomycin resistance during construction of the gene deficient mouse strain [17]. This raises an important caveat to assigning any defect in J λ 18KO mice solely to the λ NKT cell defect. In *S. pneumoniae* infection, however, the evidence for the involvement of λ NKT cells is compelling. λ NKT cells were strongly activated in the lung to produce cytokines such as IFN γ and IL-17A within hours of infection [18], and they act to stimulate the innate immune response to protect the host within days. This rapid action is not consistent with the kinetics of a typical adaptive immune response that would be affected by altering the J λ repertoire.

λ NKT cells also play a role in host defense against bacterial infection through activation of DCs and induction of IFN γ production by CD4⁺ and CD8⁺ T cells. In *Chlamydomydia* (formerly called as *Chlamydia*) *pneumoniae* infection, λ NKT cells accumulated in the lungs in the early phase of infection and they expressed intracellular IFN γ [19]. In J λ 18KO mice, expression of CD40 and intracellular IL-12 in CD8⁺ DCs and IFN γ production by CD4⁺ and CD8⁺ T cells were reduced compared to WT mice [20]. IL-12 production by CD8⁺ DCs is known to be dependent on IFN γ and the CD40-CD40L interaction, suggesting that λ NKT cells may promote bacterial clearance by enhancing the Th1 response through stimulation of DCs with IFN γ and co-stimulatory molecules during *C. pneumoniae* infection. *Chlamydia muridarum* is the mouse model for *Chlamydia trachomatis* infection, a sexually transmitted infection that can lead to serious damage to the female reproductive tract. The results from two recent studies indicate this organism has an antigen for λ NKT cells [21, 22], although its structure is unknown. Also, infected epithelial cells degrade CD1d, suggestive of an immune evasion mechanism [23]. Despite this, there is no consensus on the role of λ NKT cells in the host response to *C. muridarum*, one report suggesting that λ NKT cells enhance the growth of this organism by inducing a Th2 cytokine milieu [19], while other studies suggest λ NKT cells can promote clearance of bacteria, but also immune pathology [22].

λ NKT cells may also play a role in preventing stroke-associated bacterial infection. Compared to WT mice, CD1dKO mice were highly susceptible to bacterial infection after transient midcerebral artery occlusion induced brain injury, a rodent model of stroke [24]. Protective liver λ NKT cell function was suppressed by noradrenergic neurotransmitter(s) after stroke. Blockade of this innervation provided protection from bacterial infection in WT mice but not in CD1dKO mice [24]. Furthermore, activation of λ NKT cells with α -GalCer induced inflammatory cytokine production and prevented bacterial infections after stroke. These data suggest that stroke-associated bacterial infections may be induced by suppression of λ NKT cell function.

Fungal infections

λ NKT cells have been shown to play an important role in host defense against several fungal infections. For example, following pulmonary infection with *Cryptococcus neoformans*, λ NKT cells accumulated in the lungs, which was dependent on the C-C motif chemokine-2 (CCL-2)/macrophage chemoattractant protein-1 (MCP-1) in the early phase of infection [25]. The Th1 response that is critical for *C. neoformans* elimination was reduced in J λ 18KO mice resulting in delayed clearance of *C. neoformans*. It was also shown that CD1dKO mice control *Aspergillus fumigatus* infection poorly [26]. λ NKT cells produced IFN γ activated by a combination of self-antigen recognition and IL-12 from antigen

presenting cells (APCs). Production of IL-12 by APCs was induced through recognition of fungal cell wall β -1,3 glucans by Dectin-1, a C-type lectin receptor that binds this glycan.

Protozoan parasite infections

λ NKT cells also participate in immune response against some protozoan parasites. For example, J 18KO mice showed increased parasite burden in skin lesions and draining lymph nodes after subcutaneous infection with *Leishmania major*, a cause of sand fly-transmitted Leishmaniasis that ranges from cutaneous lesions to visceral infection. Decreased cytokine production, NK cell cytotoxicity and increased splenic parasite burden were seen in J 18KO mice following intravenous infection, suggesting that λ NKT cells play important roles in host defense against *L. major* [27, 28]. In addition, CD1dKO mice were also shown to be more susceptible to *L. donovani* infection [29].

λ NKT cells are also involved in regulating excessive inflammation caused by parasite infection. J 18KO mice developed severe inflammation in various organs, including spleen, liver and muscles, after *Trypanosoma cruzi* infection, causing a mouse model of Chagas disease, which is characterized by chronic inflammation in heart, gastrointestinal tract and nervous system. However, CD1dKO mice developed only mild inflammation, suggesting that different NKT cell subsets, type I and type II, exert different functions. If this were true, the most simple explanation would be that type II NKT cells expressing diverse TCR have pro-inflammatory functions, with λ NKT cells having an anti-inflammatory function during *T. cruzi* infection [30]. However, because of the distortion in the J repertoire in J 18KO mice noted above, further evidence will be required to confirm the counteracting effects of different CD1d-reactive populations.

Viral infections

λ NKT cells have also been shown to respond to viral infections, particularly when herpes family viruses are involved. For example, λ NKT cells produce IFN γ within 36h of infection with mouse cytomegalovirus (MCMV), a herpes virus. This response was mediated by TLR9, IL-12 and IFN γ , but not by CD1d, showing that inflammatory signals independent of TCR engagement are sufficient in some contexts for the activation of λ NKT cells [31, 32]. IFN γ producing NK cells and IFN γ in blood were decreased in CD1dKO mice resulting in lower survival rates compared to WT mice. Interestingly, increased susceptibility to high dose MCMV infection was not seen in J 18KO mice on the B6 background, although they had lower IFN γ in blood compared to WT mice [32]. The discordant data in these two strains are subject to the interpretations discussed above. Interestingly, on the BALB/c background, J 18KO do have higher viral plaque forming units in the spleen and liver at early times after infection (A. Tyznik, M. Kronenberg, C. Benedict, unpublished data). It is possible that BALB/c mice, which lack the Ly49H activating NK receptor that is so important for the response to MCMV [33, 34], are more dependent on λ NKT cells than their B6 strain counterparts.

λ NKT cells play a protective role against genital HSV-2 infection through early production of IFN γ [35], with CD1dKO mice being more susceptible to genital HSV-2 infection. λ NKT cells also are involved in controlling herpes simplex virus type 1 (HSV-1), as demonstrated by impaired viral clearance in both CD1dKO and J 18KO mice [36]. However, the protective role of λ NKT cells in HSV-1 infection still remains uncertain, with other groups reporting similar susceptibility to HSV-1 infection between CD1dKO and WT mice [37]. Such discrepancies are common in studies of λ NKT cells in the context of other infections, including bacterial infections, for example infection with *Pseudomonas aeruginosa* [38, 39]. Discordant data have also been published for parasite infections, for example using *Plasmodium* species [40, 41]. These discrepancies may be due to differences in the strain, as

illustrated above when considering the response to MCMV, the route of infection or dose, or intrinsic differences between inbred mouse colonies due to environmental differences, particularly variability in the microbiota.

IKT cells and infectious diseases in humans

Several reports have indicated the possible important role of IKT cells in controlling viral infection in humans. IKT cells were reported to be reduced in number and function in patients with active *Mycobacterium tuberculosis* infection [42]. Considering viral infections, a girl who died after receiving the varicella vaccine was shown to have a defect in IKT cells and no other immune defects were observed [43]. Also, a life-threatening infection developed in boy given the varicella vaccine, and he was shown to be deficient for CD1d expression and IKT cells [44]. X-linked lymphoproliferative syndrome (XLP) patients have a mutation in a lymphocyte adaptor protein called signaling lymphocytic activation molecule associated protein (SAP). These individuals were shown to lack IKT cells, and the patients die from uncontrolled Epstein-Barr virus (EBV) infection [45, 46]. Furthermore, some XLP patients have a mutation in the X-linked inhibitor of apoptosis (XIAP) gene but not in SAP also lack IKT cells. While these data suggest that IKT cells may have an important role in EBV infection [47], XLP patients have other immune defects. Interestingly, several human viruses down regulate CD1d expression, including HSV-1, HIV and Kaposi sarcoma herpes virus [48] [49–52], suggestive of an immune evasion strategy. In summary, much of the data in humans are suggestive of a role for IKT cells in chronic viral infections. This is surprising considering the widely held view that IKT cells are early responders, but NK cells are also active in these infections and it is known that IKT cells can participate in NK cell activation.

IKT cells induce inflammation

As mentioned earlier in this review, IKT cells play a protective role in various microbial infections in mice. However, as noted in the discussion of *Chlamydia* infection, IKT cells can also in some contexts contribute to immunopathology. Mice infected with Sendai virus developed chronic lung disease characterized by mucous cell metaplasia and airway hypersensitivity that resembles asthma and chronic obstructive pulmonary disease (COPD) in humans [53]. Macrophages are major sources of IL-13 after Sendai virus infection, and this disease was not observed in either IL-13KO or macrophage depleted mice. Interestingly, IL-13 producing IKT cells were increased in the lung at the chronic phase of Sendai virus infection, and IKT cells were shown to directly stimulate macrophages for IL-13 production [53]. J 18KO and CD1dKO mice had reduced IL-13 producing macrophages, mucous cell metaplasia and airway hypersensitivity in lung. Moreover, IL-13⁺ macrophages and IKT cells were increased in lungs from COPD patients compared to controls [53]. These data suggest that IKT cells participate in the pathogenesis of chronic inflammatory lung disease. IKT cells also participate in several models of sterile inflammation, such as ischemia reperfusion injury, but in some of these cases the inflammation is driven by IKT cell derived IFN rather than IL-13 [54].

Although antimicrobial immune responses have been implicated in the induction of some autoimmune diseases, in most cases this has not been incontrovertibly demonstrated. Primary biliary cirrhosis (PBC) is characterized by immune-mediated destruction of the small bile ducts leading to bile extravasation, chronic inflammation and subsequent liver fibrosis. Essentially all PBC patients have signature autoantibodies against mitochondrial pyruvate dehydrogenase complex E2 (PDC-E2) that cross-react with the homologous enzyme of *Novosphingobium aromaticivorans*, a commensal bacterium in the intestine [55]. *N. aromaticivorans* infection in mice induces antibodies against microbial PDC-E2 that cross-react with the mitochondrial homologue, and that also induce chronic T cell mediated

inflammation in small bile ducts [56]. The liver inflammation can be induced in recipient mice by transfer of CD4 and CD8 T cells, but not α NKT cells obtained from mice with the established liver disease, showing that autoreactive T cells mediate the inflammation. However, α NKT cells were required for the initiation of this disease [56]. It was previously shown that α NKT cells were increased in the liver of PBC patients compared to healthy controls [57]. Collectively, these data indicate that α NKT cell response to a commensal bacterium may play an important role in initiating autoimmunity in the liver.

Intestinal microbes affect α NKT cell function

Because α NKT cells detect diverse microbes, it would seem likely that they are influenced by the content of commensal microbes. Although an early study indicated that α NKT cells were not greatly diminished in number and function in germ free mice [58], more recent studies have shown that the intestinal microbiota has some effects on α NKT cells. α NKT cells express diverse V β rearrangements along with their invariant TCR α chain, but these involve predominantly V β 8, along with smaller contributions by V β 7 and V β 2. α NKT cells isolated from specific pathogen free (SPF) mice obtained from different vendors showed differences in both the frequency of α NKT cells expressing TCR V β 7 and their cytokine response to

GalCer. Additionally, α NKT cells isolated from germ free mice had a less mature or activated phenotype, and fewer cells made cytokines including TNF, IFN γ , IL-4 and IL-13 in response to GalCer when compared to cells from SPF mice [59]. α NKT cells isolated from mice deficient in either TLR signaling or IL-12 showed a normal phenotype and antigen response similar to SPF wild type mice, suggesting that the effect of intestinal microbiota on α NKT cells is independent of inflammatory signals. Moreover, the immature phenotype of α NKT cells in germ-free mice was restored by intra-gastric injection of *Sphingomonas* bacteria, which possess α NKT cell antigens, but not *E. coli*, which lack α NKT cell antigens [59]. These data suggested that interaction of α NKT cells with certain bacteria in the intestinal microbiota is critical for these cells to acquire a fully mature phenotype and function.

Germ-free mice and SPF mice born under germ-reduced conditions were shown to have an increased accumulation of α NKT cells in the lamina propria and epithelium of the small and large intestine [59, 60] due to increased levels of the chemokine CXCL16 made by intestinal cells [60]. Germ-free mice exhibited a higher mortality in oxazolone-induced colitis [60]. Blocking CD1d with antibodies inhibited colitis, and an increased susceptibility to colitis was absent in α NKT cell deficient germ free mice. These data are consistent with earlier work showing that oxazolone-induced colitis is dependent on α NKT cells, CD1d, and IL-13 [61]. Germ-free mice also showed an α NKT cell accumulation in the lung, and these mice were more sensitive to allergic asthma [60]. Interestingly, exposure of neonatal mice, but not adult mice, to SPF conditions normalized the number of α NKT cells in the colon and lung and in protection from increased colitis and asthma. Earlier work also showed that exposure of SPF suckling mice either to influenza virus, or to a glycolipid from *Helicobacter pylori*, the cause of most stomach ulcers, also would protect from asthma later in life [62]. The *H. pylori* glycolipid was shown to activate some α NKT cells, and both the viral infection and the glycolipid caused changes in the α NKT cell population that were correlated with asthma protection. Importantly, as with the germ free mice, the exposure to microbe (or the glycolipid) had to occur early in life in order for it to be protective long-term. These data are in line with the hygiene hypothesis, and they suggest that the activation of α NKT cells early in life may play an important role in setting the inflammatory tone of the immune response.

α NKT cells recognize bacterial glycolipid antigens

α NKT cells can be activated to secrete cytokines such as IFN γ by several pathways, including engagement of their TCR by foreign antigen, engagement by self-antigen, which

usually also requires another signal delivered by a cytokine such as IL-12 from innate immune cells, a combination of cytokines alone, such as IL-12 and IL-18 in the absence of TCR engagement, or by engagement of activating NK receptors [63, 64]. Therefore, although λ NKT cells participate in the response to various microbes, it remained to be determined if they could recognize microbial glycolipid antigens with their TCR. Several microbial lipids, including phosphatidylinositol mannosides (PIMs) from *Mycobacterium bovis* bacillus Calmette Guérin, lipophosphoglycan from *Leishmania donovani*, a cholesterol-containing glycolipid from *Helicobacter pylori* and an antigen from *C. muridarum* were reported to activate λ NKT cells [21, 22, 29, 62, 65]. With the exception of the PIMs and the *H. pylori* antigen, the full structures of the antigens responsible are not known. Also, in several cases the compound in question may only activate a minority of the λ NKT cells, and because the α chain is invariant, this might reflect particular specificities imparted by the TCR rearrangement.

We and other groups have found that glycosphingolipids (GSLs) from *Sphingomonas/Novosphingobium* bacteria are essentially universal antigens for λ NKT cells [66–68]. *Sphingomonas* GSLs containing either a galacturonic acid (GalA) or a glucuronic acid (GlcA) have structures similar to GalCer (Figure 1). The *in vitro* experiments showed that the GSLs purified from *Sphingomonas* bacteria bound to CD1d and stimulated mouse λ NKT cells to produce cytokines in a CD1d dependent manner. Both purified and synthetic *Sphingomonas* GSLs induced cytokine production by λ NKT cells *in vivo*. The λ NKT cell activation by *Sphingomonas* GSLs was also observed in mice deficient in TLR signaling or IL-12, showing that this activation was induced directly by recognition of *Sphingomonas* GSLs and not indirectly by cytokines and inflammation [66, 67]. Furthermore, CD1d tetramers loaded with *Sphingomonas* GSLs recognized a majority of mouse λ NKT cells [66–68]. Human λ NKT cells from expanded lines recognized *Sphingomonas* GSLs and produced cytokines in a CD1d dependent manner, indicating the response is conserved. Furthermore, CD1d tetramers loaded with *Sphingomonas* GSLs recognized essentially all of the TCR⁺ cells in several human λ NKT cell lines cells [66, 67]. *Sphingomonas* bacteria are widely distributed in the environment [69], and have been detected as a commensal organism in some individuals [55], but the predominant glycosphingolipids in different *Sphingomonas* spp are not identical, and the subtle variations in lipid and carbohydrate structure can have a big influence on antigenic potency [70, 71].

Most bacteria do not produce glycosphingolipids, but *Bacteroides* spp. are a prevalent commensal organism of the human intestine, and many of them also have the capacity to produce sphingolipids. Recently, a glycosphingolipid antigen from *Bacteroides fragilis* has been purified and characterized and it also activates mouse and human λ NKT cells [72].

We identified glycolipids from the spirochete *Borrelia burgdorferi*, the first antigens that stimulate the population of λ NKT cells that are derived from a pathogenic organism [73]. *B. burgdorferi* is a causative agent of Lyme disease, the most common tick-borne disease in North America and Europe. Lyme disease leads to diverse symptoms including inflammation in the joint, heart and nervous system. *B. burgdorferi* glycolipid-II (BbGL-II), one of two major glycolipids purified from *B. burgdorferi* [74], was shown to bind to CD1d and to stimulate mouse λ NKT cells in a CD1d dependent manner [73]. The structure of the *B. burgdorferi* antigen, called BbGL-II, is a β -galactosyl-diacylglycerol with a single β -linked hexose sugar and two hydrophobic lipid tails. It is in this way similar to the *Sphingomonas* GalAGSL or GalCer, although BbGL-II is a diacylglycerol, a different type of lipid from the ceramide lipids in glycosphingolipids (Figure 1). A synthetic version of the *B. burgdorferi* antigen, a glycolipid named BbGL-IIc, which contains a palmitic acid (C_{16:0}) and an oleic acid (C_{18:1}), two major fatty acids in *B. burgdorferi*, also stimulated the majority of mouse λ NKT cells for cytokine release both *in vitro* and *in vivo* [73]. Although

human λ NKT cells also responded to *B. burgdorferi* glycolipids and they required glycolipids with more unsaturation in the fatty acids, such as BbGL-II containing a linoleic acid (C_{18:2}) and an oleic acid (C_{18:1}).

To determine if λ NKT cells also recognize antigens from pathogenic microbes that cause widespread disease, we tested if λ NKT cells could recognize glycolipids from *S. pneumoniae*. This organism is estimated to cause 11% of all the deaths in children younger than 5 years old in the world [75]. As noted above, an effective host response of mice to *S. pneumoniae* is highly dependent on λ NKT cells. We found that λ NKT cells in the lung produce cytokines, including IFN γ and IL-17, at the early phase of pulmonary infection with *S. pneumoniae* [18]. Cytokine production of λ NKT cells was inhibited by treatment with anti-CD1d antibodies, and CD11c⁺ DCs purified from *S. pneumoniae* infected mice stimulated mouse λ NKT hybridomas for cytokine production. These data suggested that λ NKT cells recognize antigens that were induced after *S. pneumoniae* infection. To identify the antigen that λ NKT cells recognize during *S. pneumoniae* infection, we isolated two major glycolipids that are diacylglycerols containing either monosaccharide of glucose or disaccharides of galactose and glucose, and fatty acids of palmitic acid and vaccenic acid; vaccenic acid is rare in mammalian cells. The glucosyl-diacylglycerol (Glc-DAG) isolated from *S. pneumoniae* induced cytokine production by several λ NKT hybridomas, but not hybridomas from type 2 NKT cells, in a CD1d dependent manner [18]. Like the other antigens identified, the *S. pneumoniae* Glc-DAG also induced cytokine production by λ NKT cells *in vivo* independent of TLR signaling and IL-12. By testing several synthetic versions of *S. pneumoniae* Glc-DAG containing different fatty acids, we determined that a synthetic version of the natural compound, which is Glc-DAG-s2 containing a palmitic acid in *sn*-1 position and a vaccenic acid in *sn*-2 position of the glycerol, is required (Figure 2). Therefore, although the lipid tails are buried in the CD1d groove, there are precise structural requirements for the lipid because this determines not only the ability to bind to CD1d effectively, but also the orientation of the sugar that protrudes from the groove and which forms a major portion of the epitope recognized by the λ NKT cell TCR. Human λ NKT cells also produced cytokines in response to the purified *S. pneumoniae* Glc-DAG and Glc-DAG-s2. Interestingly, group B *Streptococcus* (GBS), a leading causative agent of meningitis and sepsis in newborns has an antigen identical to Glc-DAG that stimulates λ NKT cells. These data show that λ NKT cells recognize glycolipid antigens of certain pathogenic Gram-positive bacteria including *S. pneumoniae* and GBS.

λ NKT cell TCR-mediated recognition of these bacterial glycolipid antigens, including GalA-GSL, BbGL-IIc and Glc-DAG-s2, was confirmed by binding studies using surface plasmon resonance (SPR), and also by determining the crystal structures of ternary complex of CD1d/bacterial antigen/ λ NKT cell TCR [76, 77]. By comparing the structures of glycolipid antigen complexes with mouse CD1d, before and after TCR binding, we showed that λ NKT cell TCR binding caused a marked conformational change in the sugar and CD1d reorienting them to a conserved positioning that closely resembles the GalCer complex with CD1d [18, 76, 77, 78]. These data demonstrate that λ NKT cell TCR can reorient bacterial antigens to a preferred position for a conserved interaction.

Recognition of bacterial antigens by λ NKT cells contributes to defense against bacterial infection

λ NKT cells produce IFN γ and IL-4 in the early phase of tick bite-infection with *B. burgdorferi* [79]. Most infected J λ 18KO BALB/c mice could not eliminate bacteria from their tissues, and they exhibited more severe joint inflammation compared to WT mice. J λ 18KO C57BL/6 mice also showed increased bacterial burden and inflammation in the heart compared to WT controls after *B. burgdorferi* infection [80]. On the C57BL/6

background, λ NKT cells accumulated in the heart and produced IFN γ , that is important for bacterial clearance after *B. burgdorferi* infection. IFN γ stimulated macrophages to increase cytokine production and phagocytosis of *B. burgdorferi* spirochetes [80]. Furthermore, the λ NKT cell response to *B. burgdorferi* in the liver was visualized directly by intravital spinning-disk microscopy. Following intravenous *B. burgdorferi* infection, Kupffer cells in the liver sinusoids captured the bacteria [81]. λ NKT cells patrolling the liver sinusoids [82] formed a cluster on the *B. burgdorferi* injected Kupffer cells which led to IFN γ production and elimination of bacteria in a CD1d and CXCR3 dependent manner [81]. In the absence of λ NKT cells, *Borrelia* spirochetes were greatly increased in the joint tissue. Collectively, these findings suggest that λ NKT cells can enhance bacterial clearance and prevent inflammation in the joint and heart of *B. burgdorferi* infected mice through recognition of bacterial antigens.

J18KO and CD1dKO mice also are more susceptible to *S. pneumoniae* infection [15, 83]. We tested if blocking antigen recognition by λ NKT cells would affect bacterial clearance. Bacterial loads in lungs were significantly increased in mice treated with anti-CD1d antibodies compared to controls [18]. These data show that recognition of glycolipid antigens by the λ NKT cell TCR plays an important role in the clearance of *S. pneumoniae*. It was also shown that IL-12 presumably from TLR stimulated APCs is important for IFN γ production by λ NKT cells during *S. pneumoniae* infection [18, 83]. These data have contributed to the hypothesis that self-antigen presentation is important even in those infections, such as *S. pneumoniae*, in which the invading microbe expresses a foreign antigen. While the recognition of foreign and self-antigens are not mutually exclusive, there is no reason why the need for an additional signal provided by IL-12 cannot apply equally to foreign as well as self antigens. Furthermore, it seems unlikely that abundant glycolipid antigens for λ NKT cells, amounting to more than 40% of the lipids in the case of *S. pneumoniae*, do not participate in λ NKT cell activation. Collectively, the data suggest that recognition of bacterial glycolipid antigens by λ NKT cells is very useful to detect certain bacteria in addition to the classical TLR mediated detection of microbial associated molecular patterns. Recognition of these bacteria by the invariant TCR of λ NKT cells is conserved between rodents and mammals, indicating that λ NKT cells are evolutionally conserved because these cells are important in detecting certain pathogenic microbes.

Conclusions

Effective vaccines are useful for preventing infectious diseases. Glycolipid mediated λ NKT cell activation induces stimulation of DCs and other innate immune cells, including NK cells, leading to enhanced acquired immunity (Figure 2). Several reports indicate that λ NKT cell activation may be applied to induce effective adjuvant activity. Intranasal co-administration of GalCer and either influenza vaccine or inactivated virus induced protection of mice from lethal challenge with influenza [84–86]. The mice vaccinated with GalCer and either influenza vaccine or inactivated virus also exhibited increased antibody titers and generation of memory CTL [84–87]. Glycolipid mediated λ NKT cell activation has also been shown to enhance the protective effect of vaccines against malaria and genital HSV-2 infection [88, 89]. Therefore we suggest that the adjuvant effect mediated by λ NKT cells may be applicable to vaccines for various infections and may be useful for designing new types of vaccines for humans.

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Figure 1. Structures of the glycolipid antigens for *i*NKT cells

Structures of GalCer, galacturonic acid (GalA) containing glycosphingolipid (GalAGSL) and glucuronic acid (GlcA) containing GSL (GlcAGSL) from *Sphingomonas* bacteria, *Borrelia burgdorferi* glycolipid (BbGL)-IIc, and glucosyl-diacylglycerol (Glc-DAG-s2) from *Streptococcus pneumoniae* are shown. The asterisk indicates that the 2-hydroxyl on the acyl chain of GalAGSL is sometimes present in bacteria.

Figure 2. iNKT cell activation augments innate and acquired immunity

Upon activation through recognition of glycolipid antigens (Ag) presented by CD1d molecules on DCs, iNKT cells start to express CD40L and produce IFN- γ and IL-4. Subsequently, activated iNKT cells promote activation of DCs through cytokines and the CD40-CD40L interaction. In return, DCs secrete IL-12, which stimulates IFN- γ production by iNKT cells and NK cells, leading to further stimulation of DCs in a positive feedback loop. DC activation induces maturation leading to enhanced priming of CD4 and CD8 T cells. CD4 T cells provide help to B cells for antibody production. Collectively, iNKT cell activation augments both innate and acquired immunity.