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## Invariant natural killer T cells: front line fighters in the war against pathogenic microbes

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### Abstract

Invariant natural killer T ( $\lambda$ NKT) cells constitute a unique subset of innate-like T cells that have been shown to have crucial roles in a variety of immune responses.  $\lambda$ NKT cells are characterized by their expression of both NK cell markers and an invariant T cell receptor (TCR)  $\alpha$  chain, which recognizes glycolipids presented by the MHC class I-like molecule CD1d. Despite having a limited antigen repertoire, the  $\lambda$ NKT cell response can be very complex, and participate in both protective and harmful immune responses. The protective role of these cells against a variety of pathogens has been particularly well documented. Through the use of these pathogen models, our knowledge of the breadth of the  $\lambda$ NKT cell response has been expanded. Specific  $\lambda$ NKT cell antigens have been isolated from several different bacteria, from which  $\lambda$ NKT cells are critical for protection in mouse models. These responses can be generated by direct, CD1d-mediated activation, or indirect, cytokine-mediated activation, or a combination of the two. This can lead to secretion of a variety of different Th1, Th2, or Th17 cytokines, which differentially impact the downstream immune response against these pathogens. This critical role is emphasized by the conservation of these cells between mice and humans, warranting further investigation into how  $\lambda$ NKT cells participate in protective immune responses, with the ultimate goal of harnessing their potential for treatment.

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Invariant natural killer T ( $\lambda$ NKT) cells are a unique subset of innate-like T lymphocytes that have gained increasing interest over the last 20 years due to their potent immunostimulatory potential. While they were initially discovered in mice, it was found that the development and specificity of these cells is highly conserved throughout mammalian evolution, including humans (Koseki et al. 1990; Porcelli et al. 1993; Brossay et al. 1998). Thus, the many studies carried out in rodent model systems could have potential benefits for humankind. This has encouraged further studies to understand how  $\lambda$ NKT cells are naturally activated, with the ultimate goal of harnessing their therapeutic potential.

$\lambda$ NKT cells are members of a class of natural lymphocytes that bridge the innate and adaptive immune system, and which can be actual participants in the innate immune response (Nagarajan and Kronenberg 2007). In contrast to mainstream T cell antigen receptor (TCR)  $\alpha\beta$  CD4 and CD8 T lymphocytes, with diverse antigen receptor

rearrangements,  $\lambda$ NKT cells have an invariant TCR $\alpha$  chain. In mice, this is formed by an invariant V $\alpha$ 14-J $\alpha$ 18 rearrangement (*Trav11-Traj18*) (Dellabona et al. 1994; Lantz and Bendelac 1994), while humans have a homologous invariant V $\alpha$ 24 rearrangement (*Trav10-Traj18*) (Porcelli et al. 1993; Dellabona et al. 1994). The invariant TCR $\alpha$  chain, combined with the use of relatively few V $\beta$  segments, strongly limits the diversity of antigens recognized by these cells. In stark contrast to the mainstream CD4 and CD8 T cells that respond to peptides presented by major histocompatibility complex (MHC)-encoded antigen presenting molecules, the  $\lambda$ NKT TCR recognizes glycolipids and phospholipids bound to class I-like CD1d molecules expressed by a variety of cell types, including antigen presenting cells (APCs) (Koseki et al. 1990; Porcelli et al. 1993; Brossay et al. 1998). When the TCR is engaged,  $\lambda$ NKT cells are rapidly activated, and they secrete cytokines, including those typically secreted by Th1, Th2, and Th17 CD4<sup>+</sup> T cells.

Due in part to their antigen experienced phenotype, evident even in the thymus, it was originally believed  $\lambda$ NKT cells only recognized self-antigens. This idea was also based on early data that showed  $\lambda$ NKT cells were reactive to autologous splenocytes and to lipid extracts from tumor cells presented by CD1d, although specific antigens were not yet identified (Dellabona et al. 1994; Lantz and Bendelac 1994; Bendelac et al. 1995; Cardell et al. 1995; Gumperz et al. 2000). However, with the development of new tools and mouse models, the emphasis has since partially shifted because the response of  $\lambda$ NKT cells to microbial antigens is well documented.

Despite their limited TCR repertoire, the activation and responses of  $\lambda$ NKT cells are actually quite variable, depending on the stimuli, consistent with a potential role of  $\lambda$ NKT cells in a variety of downstream immune responses. In fact, many functions have been attributed to  $\lambda$ NKT cells, including immune regulation to prevent autoimmunity, tumor immune surveillance, and regulation of adipose tissue and the metabolic state. Additional roles include responses that cause asthma and some forms of autoimmunity, and exacerbation of sterile inflammatory responses (Godfrey and Kronenberg 2004; Wu and Kaer 2009; Santodomingo-Garzon and Swain 2011). In our view, however, the function of  $\lambda$ NKT cells that has been most clearly and consistently demonstrated is the role of these cells in host defense from bacterial, fungal and viral infections. The few exceptional cases, in which  $\lambda$ NKT cell responses were not protective from infections, were most likely instances of immune pathology in which excessive  $\lambda$ NKT cell activity contributed to tissue damage. The role of  $\lambda$ NKT cells as rapid responders, and as fighters against pathogenic microbes, is therefore the topic of this article. Characteristics of the investigated pathogens are described in Table 1. Several excellent reviews have appeared on this topic recently (Kinjo et al. 2013; Van Kaer et al. 2015; Zajonc and Girardi 2015), and in this review we will further describe how using these pathogen models has granted us a better understanding of the complexity and importance of the  $\lambda$ NKT cell response.

## Tools for studying $\lambda$ NKT cells

Intensive study of  $\lambda$ NKT cells in the context of infections and other immune responses has been aided by the development of critical tools, including glycolipid antigens that activate

these cells, tetramers and antibodies with bright fluorescence that allow for their detection without prior enrichment, and mouse strains with a defect in generating  $\lambda$ NKT cells.

### **$\alpha$ GalCer: a potent $\lambda$ NKT cell activator**

When  $\lambda$ NKT cells were initially discovered, specifically activating them was a challenge because their cognate antigens were unknown. The discovery of  $\alpha$ -galactosylceramide ( $\alpha$ GalCer) completely changed the way  $\lambda$ NKT cells could be studied.  $\alpha$ GalCer is a glycolipid that was originally extracted from the Okinawan marine sponge *Agelas mauritianus*. After its potent antitumor and immunostimulatory effects were revealed, Kobayashi *et al.* chemically synthesized a structurally and functionally similar compound for further investigations (Kobayashi *et al.* 1995). Subsequent *in vitro* studies using mouse cells found that  $\alpha$ GalCer is presented by CD1d molecules on APCs, and it proved to be a high-binding, potent, and specific activator of  $\lambda$ NKT cells (Kawano *et al.* 1997; Burdin *et al.* 1998). Notably, when bound to mouse CD1d, the  $\alpha$ GalCer-CD1d complex interacted with the  $\lambda$ NKT cell TCR with an affinity in the nM range (Naidenko *et al.* 1999; Benlagha *et al.* 2000; Cantu *et al.* 2003; Yu *et al.* 2005), which is stronger binding than most peptide antigens presented by MHC I or II molecules to CD8 or CD4 T cells.

Recognition of  $\alpha$ GalCer presented by dendritic cells (DCs) led *in vitro* to a robust and rapid, innate-like response, which included proliferation and cytotoxicity by V $\alpha$ 14  $\lambda$ NKT cells. This corresponded with an increase in IFN- $\gamma$  and IL-4 cytokine production (Kawano *et al.* 1997; Burdin *et al.* 1998). *In vivo* mouse analyses of the  $\alpha$ GalCer response revealed additional roles for  $\lambda$ NKT cells, including rapid (within 3–24 hours) secretion of a variety of Th1, Th2, and Th17 cytokines; as well as activation of many cell types, including DCs, NK cells, B cells, and memory CD8<sup>+</sup> and CD4<sup>+</sup> T cells (Carnaud *et al.* 1999; Singh *et al.* 1999; Burdin *et al.* 1999). Despite this broad immune modulatory potential, it was notable that in some studies  $\alpha$ GalCer tended to skew the immune response towards a Th2 phenotype (Singh *et al.* 1999; Burdin *et al.* 1999). Furthermore, upon re-exposure to  $\alpha$ GalCer, the  $\lambda$ NKT cell population acquired some properties typical of anergic T cells, including expression of PD-1 and an increased percentage of  $\lambda$ NKT cells that produce IL-10 (Parekh *et al.* 2005; Sag *et al.* 2014). Altogether, these changes suggest a potential caveat regarding the use of this unnaturally high-binding antigen for inducing prolonged Th1 responses.

Regardless of its physiologic significance, because of its high affinity for the  $\lambda$ NKT cell TCR when bound to CD1d,  $\alpha$ GalCer, and antigens closely related to it, have proven very useful in the formation of CD1d tetramers, which can be used to precisely identify and purify  $\lambda$ NKT cells in various situations. An antibody to the complementarity-determining region 3 (CDR3) of the invariant TCR $\alpha$  chain also can be used to define this population in humans, although despite the relatively high degree of CDR3 $\alpha$  conservation, an antibody with a similar specificity is not available for studies in rodents. These tools have been critical in the studies described below.

While studies using  $\alpha$ GalCer began to give answers as to what  $\lambda$ NKT cells could do if they were pharmacologically activated, questions remained regarding how activation of  $\lambda$ NKT cells by an exogenous antigen benefits the host. To begin to address this,  $\alpha$ GalCer was utilized in the context of infections in mice. Administration of  $\alpha$ GalCer prior to pathogen

challenge improved disease outcomes in several experimental infection models, including infections with rodent malaria parasites *Plasmodium yoelii* or *Plasmodium berghei*, or by the fungal pathogen *Cryptococcus neoformans* (Gonzalez-Aseguinolaza et al. 2000; Kawakami et al. 2001). Additionally, post-infection treatment of mice with  $\alpha$ GalCer increased survival and promoted clearance of *Pseudomonas aeruginosa* (Nieuwenhuis et al. 2002) and *Mycobacterium tuberculosis* (Chackerian et al. 2002). These data suggest that  $\mathcal{N}$ KT cells could have protective roles if they were artificially stimulated, and that optimized glycolipid antigens could serve as vaccine adjuvants (Ko et al. 2005; Li et al. 2010; Carreño et al. 2014), but they did not fully explicate how  $\mathcal{N}$ KT cells were involved in protection, and under what circumstances they are naturally activated.

### Mouse models of $\mathcal{N}$ KT cell deficiency

While initial  $\alpha$ GalCer studies showed the impact of activating  $\mathcal{N}$ KT cells, they didn't answer how the host is impacted by the absence of  $\mathcal{N}$ KT cells. To tackle this question,  $\mathcal{N}$ KT cell-deficient mouse models were developed, including *CD1d*<sup>-/-</sup> and *J $\alpha$ 281KO* (*Ja18*<sup>-/-</sup> or *Tra18*<sup>-/-</sup>) mice. Reported strains of *CD1d*<sup>-/-</sup> mice lack either *Cd1d1* or both the *Cd1d1* and *Cd1d2* loci, and thus cannot efficiently positively select  $\mathcal{N}$ KT cells during thymus differentiation (Smiley et al. 1997; Chen et al. 1997). One caveat to using these mice is that in addition to lacking  $\mathcal{N}$ KT cells, sometimes called type 1 NKT cells, *CD1d* deficient mice also lack type 2 NKT cells. These are *CD1d*-dependent cells with a more diverse TCR repertoire, and which have different and more diverse specificities compared to  $\mathcal{N}$ KT cells. A similar caveat applies to experiments using *in vivo* treatment with blocking anti-*CD1d* mAbs.

Alternatively, *Ja18*<sup>-/-</sup> mice lack the *Tra18* gene segment and therefore cannot form the invariant TCR $\alpha$  chain necessary for  $\mathcal{N}$ KT development (Kawano et al. 1997). The original strain of these mice also had decreased rearrangement of all the *J $\alpha$*  segments upstream of *Tra18* (Bedel et al. 2012). This is presumably because the neomycin resistance gene cassette, incorporated during construct generation, was not removed, and its transcription in the opposite orientation from the  $\alpha$  chain genes interfered with rearrangement of the upstream *J $\alpha$*  segments (Bedel et al. 2012). A new *Ja18*<sup>-/-</sup> mouse, with the neomycin resistance gene removed, and consequently a normal repertoire of *J $\alpha$*  segment rearrangements, addressed this problem. This model strain has yet to be widely tested in different model systems (Chandra et al. 2015). Despite their various limitations, in tandem with pathogen challenges, all of these models have been highly useful for understanding what roles  $\mathcal{N}$ KT cells have in the immune response, and why they are so important for host survival. Of human relevance, at least one person deficient for *CD1d* has been described, and this individual had a disseminated varicella infection after receiving the varicella vaccine to prevent chicken pox (Leung et al. 2014). The vaccine contains a live, attenuated virus, further suggesting the importance of  $\mathcal{N}$ KT cells for human health, particularly in the context of infections.

## **̳NKT cells help protect the host from infections**

### **iNKT cell-mediated protection from pathogenic bacteria**

An important step required for understanding the importance of ̳NKT cells in fighting infection has been to determine if the absence of these cells leads to host susceptibility. Infection with *Borrelia burgdorferi*, the spirochete that causes Lyme disease, was among the first pathogens with an antigen for ̳NKT cells that demonstrated that the absence of ̳NKT cells has detrimental effects. CD1d deficiency rendered otherwise disease-resistant mice susceptible to *B. burgdorferi*-induced arthritis (Kumar et al. 2000). Later studies in *Ja18<sup>-/-</sup>* mice confirmed ̳NKT cell deficiency resulted in greater and more persistent joint swelling than in wild type BALB/c mice (Tupin et al. 2008) and increased carditis on the C57BL/6 genetic background (Olson et al. 2009). This inflammation and disease correlated with systemic increases in the spirochetes, particularly in the liver, joints, and bladder in BALB/c mice (Lee et al. 2010). Altogether these findings suggest that ̳NKT cells play a role in controlling the disease burden in this infection, perhaps through increased IFN-̳ secretion (Olson et al. 2009).

Likely the most striking impact of ̳NKT cell deficiency has been shown in the context of pulmonary *Streptococcus pneumoniae* infection. Whereas 75% of wild type mice survived pulmonary infection, 87.5% of ̳NKT cell-deficient *Ja18<sup>-/-</sup>* mice were dead by day 7 (Kawakami et al. 2003). This correlated with a dramatic increase in bacterial loads at this time point (Kawakami et al. 2003). Several other models of infection have demonstrated that ̳NKT cell deficiency led to increased bacterial loads, particularly *P. aeruginosa* (Nieuwenhuis et al. 2002; Hazlett et al. 2007), *M. tuberculosis* (Sada-Ovalle et al. 2008), and *Chlamydia pneumoniae* (Joyee et al. 2007).

### **Mechanisms for activating iNKT cells**

The use of  $\alpha$ GalCer demonstrated that the ̳NKT TCR recognizes lipids bound by CD1d molecules, and the cells thereby become activated. Use of  $\alpha$ GalCer, however, was not informative as to whether direct antigen presentation is always both necessary and sufficient for ̳NKT cell activation in the context of bacterial infections.

In support of the direct presentation model, in *P. aeruginosa* infection of wild type mice, blocking CD1d with an antibody significantly diminished bacterial clearance from the lungs (Nieuwenhuis et al. 2002). This result was similar to the diminished clearance observed after infection of *Cd1d<sup>-/-</sup>* mice. Comparable effects of anti-CD1d antibodies also were seen in mice infected with *S. typhimurium* or *Staphylococcus aureus*, which also showed an increase in disease susceptibility after CD1d blockage (Brigl and Brenner 2004). The use of Nur77GFP reporter mice, in which T lymphocytes express GFP after TCR stimulation, provided further proof of the importance of CD1d-mediated activation of the ̳NKT cell TCR. With this model, ̳NKT cells became GFP positive and produced cytokines in response to *S. pneumoniae* and *Sphingomonas paucimobilis*, microbes that have antigens that stimulate ̳NKT cells, but not in response to **mouse cytomegalovirus (MCMV)** or *Salmonella typhimurium* (Holzapfel et al. 2014). This agrees with data indicating that MCMV responses by ̳NKT cells do not require TCR engagement by either a self or foreign

antigen (Wesley et al. 2008; Tyznik et al. 2014), however, the result using the TCR reporter mice appears to contradict the earlier mentioned *S. typhimurium* data, where CD1d blockade negatively impacted  $\lambda$ NKT cell activation (Brigl et al. 2003).

In many cases in which a protective response required antigen presentation to the  $\lambda$ NKT TCR, it remained uncertain if the antigen was of pathogen or host origin. Several  $\lambda$ NKT glycolipid antigens of bacterial origin have been identified. Among the first, Fisher *et al* identified a phosphatidylinositol mannoside from the **mycobacterial** cell wall, which induced  $\lambda$ NKT cell IFN- $\gamma$  release and cytotoxicity in a CD1d-dependent manner (Fischer et al. 2004). A later study with synthetic versions of these antigens did not confirm  $\lambda$ NKT cell reactivity to these compounds (Kinjo et al. 2006). Since then, several other microbial glycolipid antigens have been isolated and tested, including *S. paucimobilis* glycosphingolipids, which activated mouse hybridomas to produce IL-2 and human  $\lambda$ NKT cell lines to produce IL-4 and IFN- $\gamma$  (Wu et al. 2005). The ability of these *Sphingomonas* glycosphingolipids to activate  $\lambda$ NKT cells was further shown in mouse cell lines and *in vivo* studies (Kinjo et al. 2005; Mattner et al. 2005). A glycosphingolipid antigen for  $\lambda$ NKT cells also has been found in *Bacteroides fragilis*, a commensal organism in mice (Wieland Brown et al. 2013). This antigen has immune modulatory properties, because when germ free mice are exposed to it early in life, they were more resistant to an IL-13-driven model of colitis (An et al. 2014). Sponges are associated with many bacterial species, and the finding of microbial antigens highly similar to  $\alpha$ GalCer in *B. fragilis* is consistent with a microbial origin for  $\alpha$ GalCer, and with the widely held view that the marine sponge-derived antigen actually originated from microbes that were associated with the sponge.

Diacylglycerol-containing glycolipids were found to be the primary  $\lambda$ NKT antigens in *Borrelia burgdorferi* (Kinjo et al. 2006), *S. pneumoniae*, and **Group B streptococcus** (*Streptococcus agalactiae*) (Kinjo et al. 2011). Thus, these descriptions and others have shown that several bacteria contain  $\lambda$ NKT cell antigens in their cell walls, and it is likely that these antigens are presented by CD1d in the course of natural infection. Additionally, it has been shown that *Helicobacter pylori* contains cholesteryl  $\alpha$ -glucoside antigens that activate  $\lambda$ NKT cells (Ito et al. 2013). These antigens are markedly different in structure, and moreover they are composite of microbial and host material, with the mammalian host providing the cholesterol moiety. The biochemical basis for the recognition of the cholesterol-containing antigens is much less well defined than it is for the glycosphingolipids and glycosylated diacylglycerol antigens.

Despite this evidence for the existence of microbial antigens, including biochemical and structural evidence that the  $\lambda$ NKT cell TCR engages them when presented by CD1d (Kinjo et al. 2006; Zajonc et al. 2006; Wang et al. 2010; Li et al. 2010; Girardi et al. 2011), others have suggested the response to *S. typhimurium* and to many other bacteria involves the recognition of the elusive self-antigen(s) for  $\lambda$ NKT cells; the structure of these self-antigens is still a source of controversy (Mattner et al. 2005; Mallevaey et al. 2011; Brennan et al. 2011). Self-antigen recognition was revealed most consistently in the context of IL-12 (Brigl et al. 2011; Brennan et al. 2011). If this were true, then in *S. typhimurium* infected mice, the TCR signal from the self-antigen must have been below the threshold for detection in the Nur77GFP reporter mice, because no TCR signal could be detected. Alternatively, it is

possible that the anti-CD1d blocking antibody treatment was effective because it elicited a cytokine response due to CD1d cross linking that was immune suppressive (Colgan et al. 1999; Brigl et al. 2003). Despite these unresolved issues, the data described above confirm that CD1d antigen presentation and TCR stimulation were necessary in a number of contexts for an  $\lambda$ NKT cell response to some infectious bacteria, however, limited data exists on which APC must express CD1d and present the antigen.

Intravital imaging has granted the capability to directly look at interactions between APCs and  $\lambda$ NKT cells in several tissues. *B. burgdorferi* clearance was shown to be due to direct CD1d-dependent interactions between  $\lambda$ NKT cells and Kupffer cells in the liver sinusoids that have taken up *B. burgdorferi*. These interactions led to  $\lambda$ NKT cell movement arrest and the formation of cell clusters in the liver sinusoids, an effect that was not seen in Kupffer cell-depleted or *Cd1d*<sup>-/-</sup> mice (Lee et al. 2010). Interestingly, injection of  $\alpha$ GalCer also did not induce formation of  $\lambda$ NKT-Kupffer cell clusters, further illustrating the limits of using  $\alpha$ GalCer as a model for the responses of  $\lambda$ NKT cells to infectious agents.

In addition to direct CD1d-mediated activation, many studies have also revealed the critical roles of cytokines in activating  $\lambda$ NKT cells. Analyses of several bacterial infections in mice, including *S. typhimurium*, *S. aureus*, *S. paucimobilis*, and *S. pneumoniae*, revealed that IL-12 production by dendritic cells was also necessary for  $\lambda$ NKT cell activation and production of IFN- $\gamma$  (Brigl and Brenner 2004; Brigl et al. 2011; Kinjo et al. 2011). Notably, this requirement for IL-12 could be  $\lambda$ NKT cell subset specific. Functional subsets of  $\lambda$ NKT cells analogous to CD4<sup>+</sup> Th1, Th2 and Th17 cells have been described, and they differ for expression of cytokine receptors and their responses to cytokines. For example, in *Escherichia coli* and *S. aureus* infection, in addition to TCR stimulation, NKT17 cells required production of IL-1 and IL-23 by dendritic cells in order to secrete IL-17 and IL-22 (Doisne et al. 2011). In ocular *P. aeruginosa* infection, in contrast, IL-12p40 production by macrophages and Langerhans cells was required for activation and IFN- $\gamma$  production by NKT1 cells (Hazlett et al. 2007). IL-12 therefore is not universally required by all  $\lambda$ NKT cells in all infections for stimulating responses to relatively low affinity antigens. Mouse  $\lambda$ NKT cells may even be activated purely by cytokines, in the absence of TCR engagement, and this has been documented most thoroughly during viral infection (Tyznik et al. 2008; Tyznik et al. 2014; Cocita et al. 2015). Activation of human  $\lambda$ NKT cells, however, may be more dependent on a recent, if not a concomitant, TCR stimulation (Wang et al. 2012).

Altogether these studies indicate that activation of  $\lambda$ NKT cells during bacterial infections is a complex process. In some cases, antigen presentation by CD1d is necessary, and either self or foreign antigens are recognized. In other instances, only indirect or cytokine-dependent mechanisms are involved. Some data suggest that both direct TCR recognition and indirect, cytokine dependent mechanisms are required for protective immunity. These mechanisms of activation could have critical roles in determining the type of  $\lambda$ NKT cell response and the downstream effects on other cell types.

### **$\lambda$ NKT cells activate the innate immune response**

Perhaps the most critical part of the  $\lambda$ NKT cell story to uncover is what steps occur after  $\lambda$ NKT cell activation that lead to host protection. *P. aeruginosa* has been an interesting and

useful model, because in addition to increased bacterial loads and decreased survival, infected *Cd1d*<sup>-/-</sup> mice also had lower neutrophil numbers, and lower levels of macrophage inflammatory protein 2 (MIP-2) expression in the lungs after 24 hours (Nieuwenhuis et al. 2002). When *P. aeruginosa* infected BALB/c mice were treated with  $\alpha$ GalCer, IFN- $\gamma$  production was stimulated, which increased phagocytosis of *P. aeruginosa* by alveolar macrophages and local TNF- $\alpha$  production. Similar effects were seen in ocular infection, where IFN- $\gamma$  production by  $\lambda$ NKT cells was critical for control of bacterial growth and inflammation control (Hazlett et al. 2007). However, for ocular infection, neutrophil infiltration has a damaging affect, so at this site, opposite to the lungs,  $\lambda$ NKT cell deficiency actually increased neutrophil recruitment (Hazlett et al. 2007). Together these findings suggest a potentially important role for  $\lambda$ NKT cells in mediating neutrophil responses for protection. Notably, in the pulmonary model of *P. aeruginosa* infection, a protective role for  $\lambda$ NKT cells was later shown to only be true in BALB/c mice, and not C57BL/6 mice (Benoit et al. 2015). This suggests different roles for the  $\lambda$ NKT subsets, as BALB/c mice have more NKT2 cells, which might suppress neutrophil recruitment when activated, while C57BL/6 mice are more NKT1 subset biased.

The protective response to *S. pneumoniae* was also largely regulated by IFN- $\gamma$  production, with evidence suggesting  $\lambda$ NKT cells produced this cytokine (Kawakami et al. 2003). IFN- $\gamma$  treatment or adoptive transfer of liver mononuclear cells to  $\lambda$ NKT cell-deficient mice decreased bacterial loads, and increased neutrophil counts and MIP-2, a neutrophil chemotactic chemokine, and TNF- $\alpha$  production. While this suggests  $\lambda$ NKT cells were producing the IFN- $\gamma$ , in this particular study they could not consistently and directly detect IFN- $\gamma$  production by  $\lambda$ NKT cells (Nakamatsu et al. 2007).

In *C. pneumoniae* infection, protection was also dependent on IFN- $\gamma$  and IL-12 production (Joyee et al. 2007). Interestingly, in this case  $\lambda$ NKT cell deficiency increased IL-4 and IL-5 production, two cytokines which have been correlated with increased mortality (Joyee et al. 2007). This is in stark contrast to pulmonary *Chlamydia muridarum* infection, where the  $\lambda$ NKT cell response was skewed towards producing IL-4 and IL-5, and was detrimental to survival (Bilenki et al. 2005). This is particularly interesting, as these two very similar pathogens both activated  $\lambda$ NKT cells, yet activation led to two very different cytokine responses and disease outcomes.

## The dark side of $\lambda$ NKT cell activity

While cytokine production and neutrophil recruitment are considered positive outcomes in the context of the above-mentioned infections, these responses did not always lead to protection. For example,  $\lambda$ NKT cells were recruited and activated in a TCR-dependent manner in *Francisella tularensis* infection (Hill et al. 2015). Similar to other infections, *Cd1d*<sup>-/-</sup> mice infected with *F. tularensis* had lower levels of neutrophil recruitment and produced lower levels of MCP-1, TNF- $\alpha$ , and IFN- $\gamma$  (Hill et al. 2015). However, in this case approximately 80% of *Cd1d*<sup>-/-</sup> mice survived challenge, compared to less than 20% of wild type mice, indicating the  $\lambda$ NKT cell-dependent inflammatory response could actually be detrimental to the host following infection with *F. tularensis* (Hill et al. 2015). These investigators also monitored adaptive immune responses and iBALT formation, and the lack

of  $\lambda$ NKT cells actually increased B and T cell lung infiltration, as well as the accumulation of dendritic cells, which together with lymphocytes formed the iBALT structures that correlated with protection (Hill et al. 2015). This suggests that there needs to be a balance of both innate and adaptive immune responses to protect fully from pathogens.

### Is down regulation of CD1d an immune evasion mechanism?

A test of the importance of  $\lambda$ NKT cells, or at least the importance of CD1d expression, has come from lessons we have learned from pathogens that have evolved ways to escape CD1d-mediated presentation. Several prominent examples derive from studies of viral infections, although as noted above, the  $\lambda$ NKT cell response to some viruses may not be CD1d-dependent. In one report, CD1d levels were measured in both untreated and treated individuals infected with **human immunodeficiency virus (HIV)-1**. Untreated patients had significantly lower levels of CD1d expressed on CD14+ monocytes, and this correlated with an increase in viral load in these patients (Hage et al. 2005). The HIV nef protein enhanced CD1d internalization and inhibited its transport back to the cell surface, thereby preventing further activation of  $\lambda$ NKT cells (Li and Xu 2008). HIV has also been found to directly and preferentially infect and kill  $\lambda$ NKT cells (Li and Xu 2008).

Similarly, **Herpes simplex virus (HSV)** has evolved its own mechanism of rapid, efficient down regulation of CD1d surface expression. In this case, it does not appear that HSV increased endocytosis of CD1d, but instead it redistributed endocytosed CD1d to lysosomal membranes, and prevented newly synthesized CD1d from translocating to the extracellular membrane (Yuan et al. 2006; Raftery et al. 2006; Liu et al. 2013). The importance of CD1d presentation for  $\lambda$ NKT cell functionality was reflected by impaired IFN- $\gamma$ , IL-4, and IL-10 production by  $\lambda$ NKT cells stimulated with infected DCs with reduced CD1d expression (Raftery et al. 2006).

Bacteria also have evolved what are likely immune evasion mechanisms based on CD1d down-regulation. In contrast to HIV-1 and HSV, *Chlamydia trachomatis* degrades CD1d in infected epithelium through its own chlamydial proteasome-like activity, which interacts with CD1d and causes its degradation (Kawana et al. 2006). By a completely different mechanism, *Bacillus anthracis* lethal toxin binds  $\lambda$ NKT cells and disrupts their TCR signaling, causing them to become functionally anergic (Joshi et al. 2009).

### Summary

There are a number of innate-like lymphocyte populations, but  $\lambda$ NKT cells have been particularly well studied, in part because of excellent tools that allow for the precise identification and antigen specific activation of these lymphocytes. Because they rapidly secrete many types of cytokines,  $\lambda$ NKT cells are truly multi-functional cells that play a role in a variety of immune and inflammatory responses. Their roles in the defense from infections, however, especially the responses to pathogenic and commensal bacteria, are particularly prominent. During infections  $\lambda$ NKT cells can be activated by microbial glycolipids, by enhanced presentation by CD1d of self-antigens, and/or by cytokines such as IL-12 from myeloid cells. The activated  $\lambda$ NKT cells then are able to stimulate a variety of

cell types, with the attraction of neutrophils to the infection site a key aspect of host protection in several contexts. The intense stimulation of the immune system following  $\lambda$ NKT cell activation in mice has a downside, however, as it may lead to immune pathology. Despite this potential drawback, controlled activation of  $\lambda$ NKT cells by glycolipids is currently being explored as a way to stimulate beneficial immune responses to vaccines or cancers. The concept underlying these efforts is that all humans have  $\lambda$ NKT cells with the same specificity, and that glycolipids that stimulate  $\lambda$ NKT cells could serve as adjuvants. While this is well established in mice, additional research will be required to determine if this is the case in humans.

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Table 1

Characteristics of bacteria known to activate  $\gamma$ NKT cells

BACTERIA SPECIES	GENERAL CHARACTERISTICS	PRIMARY INFECTION SITE	ASSOCIATED DISEASES	CITED PROTECTION MECHANISMS	$\gamma$ NKT CELL ACTIVATION MECHANISMS	REFERENCES
<i>Pseudomonas aeruginosa</i>	Gram negative, aerobic, mobilized with flagella	Lungs, kidneys, eyes	Pneumonia, keratitis, urinary tract infections, bacteremia, inflammation, sepsis	Alveolar macrophage-secreted chemokines and cytokines, neutrophils, $\gamma$ NKT cells	CD1d-dependent, unknown antigen plus IL-12	(Nieuwenhuis et al. 2002; Koh et al. 2009; Benoit et al. 2015)
<i>Mycobacterium tuberculosis</i>	Impervious to gram staining, intracellular (mononuclear phagocytes), rod-shaped	Lungs	Tuberculosis	Th1-polarized T cell responses (IFN- $\gamma$ ), GM-CSF production, $\gamma$ NKT cells	CD1d-dependent, self antigen plus IL-12	(Sharma et al. 2002; Chackerian et al. 2002; Tang et al. 2004; Sada-Ovalle et al. 2008; Cooper 2009; Brigl et al., 2011; Rothchild et al. 2014)
<i>Borrelia burgdorferi</i>	Double membrane capsule, spirochete	Blood, liver, joints, nervous system, heart	Lyme disease	Th1 adaptive immune responses, $\gamma$ NKT cells	CD1d-dependent, foreign antigen	(Kumar et al. 2000; Kinjo et al. 2006; Tupin et al. 2008; Olson et al. 2009; Lee et al. 2010; Oosting et al. 2016)
<i>Streptococcus pneumoniae</i>	Gram positive, $\alpha$ -hemolytic, facultative anaerobic	Lungs	Pneumonia, meningitis	Alveolar macrophages, neutrophils, antibodies, $\gamma$ NKT cells	CD1d-dependent, foreign or self antigen plus IL-12	(AlonsoDeVelasco et al. 1995; Kawakami et al. 2003; Nakamatsu et al. 2007; Brigl et al. 2011; Kinjo et al. 2011; Bai et al. 2013; Holzappel et al. 2014)
<i>Chlamydia pneumoniae</i>	Gram negative, obligate intracellular	Lungs	Pneumonia	CD8+ T cells, Th17/IL-17 with Th1, $\gamma$ NKT cells	Unknown antigen plus IL-12	(Joyce et al. 2007; Sudhanshu Shekhar 2015)
<i>Chlamydia muridarum</i>	Gram negative, obligate intracellular	Lungs, genital tract	Pharyngitis, bronchitis, pneumonitis	CD4+ T cells, $\gamma$ NKT cells	CD1d-dependent, foreign antigen	(Bilenki et al. 2005; Jiang et al. 2012; Sudhanshu Shekhar 2015)
<i>Salmonella typhimurium</i>	Gram negative, intracellular	Intestinal lumen, mesenteric lymph node, liver, spleen	Gastroenteritis	Macrophages, neutrophils, IFN- $\gamma$ , IL-4, $\gamma$ NKT cells	CD1d-dependent, self antigen plus IL-12 or cytokine alone	(Kirby et al. 2002; Brigl and Brenner 2004; Bemtan et al. 2005; Hazlett et al. 2007; Holzappel et al. 2014)
<i>Sphingomonas paucimobilis</i>	Gram negative, aerobic	Highly variable	Nosocomial infections, bacteremia, pneumonia	None cited	CD1d-dependent, foreign or self antigen plus IL-12	(Hsueh et al. 1998; Wu et al. 2005; Kinjo et al. 2005; Matner et al. 2005; Hazlett et al. 2007; Holzappel et al. 2014)
<i>Staphylococcus aureus</i>	Gram positive, facultative anaerobic	Nose, respiratory tract, skin	Abscesses, bacteremia, sepsis, pneumonia, meningitis	Neutrophils, Antibodies, $\gamma$ NKT cells	CD1d-dependent, unknown antigen plus IL-12, IL-1, and IL-23	(Brigl and Brenner 2004; Kelly-Quintos et al. 2006; Doisne et al. 2011)
<i>Francisella tularensis</i>	Gram negative, facultative intracellular	Lungs	Pulmonary tularemia, sepsis	Macrophages, AIM2 inflammasome, neutrophils	CD1d-dependent, unknown antigen	(Fernandes-Alnemri et al. 2010; Hill et al. 2015)

\* Foreign or self-antigen means that there is evidence in different papers for one type of antigenic stimulus or the other. CD1d dependent, unknown antigen means there is evidence for a role for CD1d, but there is not published evidence for the involvement of a self or foreign antigen.