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Review

How invariant natural killer T cells respond to infection by recognizing microbial or endogenous lipid antigens

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ABSTRACT

Invariant natural killer T (iNKT) cells have evolved to recognize CD1d-presented lipid antigens and are known to play important roles during infection with bacterial, viral, protozoan, and fungal pathogens. The limited antigen specificity and reactivity to self- and foreign antigens distinguish iNKT cells from MHC-restricted T cells and bear similarity to innate-like lymphocytes, such as NK cells, γδ T cells, MZB and B1-B cells. This review summarizes how direct recognition of microbial lipids or synergistic stimulation by self-lipids and pro-inflammatory cytokines results in activation of these innate-like iNKT cell during infection. iNKT cell activation in the absence of foreign antigen recognition is unique for cells bearing TCRs and underscores that not only the function but also the activation mechanism of iNKT cells is innate-like, and distinct from adaptive T cells. The different pathways of activation endow iNKT cells with the ability to respond rapidly to a wide variety of infectious agents and to contribute effectively to the early immune response during infection.

1. Introduction

The immune system has evolved the capability to present lipid antigens to T cells by CD1 molecules [1]. A growing body of evidence has documented a critical role for CD1d-restricted T cells in the immune response to bacterial, viral, protozoan, and fungal infections [2–4]. Most CD1d-restricted T cells use an invariant TCRα chain (Vα14Jα18 in mice and Vα24Jα18 in humans) combined with a limited set of TCRβ chains, co-express receptors typically found on NK cells, and thus are commonly referred to as invariant natural killer T (iNKT) cells [1,5,6]. iNKT cells were initially characterized based on their auto-reactivity to CD1d molecules and recognition of endogenous CD1d-presented self-lipids by iNKT cells is critical for their development and function [1,5]. iNKT cells are also able to recognize exogenous lipid antigens, such as the widely used α-galoctosylceramide (αGalCer), initially isolated from a marine sponge based on its anti-tumor activity, and several of its analogs [7,8]. iNKT cells display an activated/memory phenotype as a result of developmental expression of the transcription factor PLZF (promyelocytic leukemia zinc finger) and appear differentiated even in the absence of prior exogenous stimulation [9,10]. This results in their ability to rapidly secrete large amounts of cytokines following primary stimulation.

Thus, iNKT cells are distinct from peptide-specific MHC class I- and class II-restricted T cells in several important properties, including their limited clonal diversity and lack of clonal expansion or generation of memory following stimulation with exogenous antigens. In contrast, their semi-invariant antigen receptor, dual specificity for self- and foreign antigens, and their differentiated effector state at baseline make iNKT cells appear more similar to other innate-like lymphocytes such as γδ T cells, marginal zone B (MZB) cells, B1-B cells, or NK cells.

How can these innate-like iNKT cells, with their restricted TCR repertoire, respond rapidly to a wide range of diverse microbial pathogens? Recent studies have revealed that their unique properties allow iNKT cells to become activated in response to microbial products and infection through distinct pathways. There are now several examples of microbe-specific CD1d-presented lipids that stimulate iNKT cells directly. In an alternative pathway, activation of iNKT cells during infection can occur without the need for direct recognition of microbial lipids. Instead, stimulation with pro-inflammatory cytokines such as IL-12, IL-18 or type I IFNs, released by antigen-presenting cells in response to microbial products or infection, in combination with recognition of CD1d-presented self-antigens, results in rapid activation of iNKT cells. Under certain circumstances, cytokine-mediated stimulation can dominate iNKT cell activation, thus reducing the need for TCR-mediated signaling. This review summarizes how illuminating the different pathways

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of activation is critical for understanding the role and function of iNKT cells during infection.

2. Direct, microbial antigen-driven pathway of iNKT cell activation during infection

Microbial lipid antigens were described early on for T cells recognizing CD1a, CD1b, and CD1c molecules, suggesting that CD1d-restricted iNKT cells may also recognize microbial lipids [1,11]. Indeed, several studies have now convincingly demonstrated the existence of microbial lipids that directly stimulate iNKT cells.

Sphingomonas spp. bacteria are a family of α-proteobacteria and a rare natural source of α-linked glycosylceramide lipids with structural similarity to α-galactosylceramide (αGalCer), the prototypical iNKT cell antigen. These bacteria were studied by several groups in search for microbial iNKT cell antigens, and were able to stimulate murine and human iNKT cells in the presence of DCs in a CD1d-dependent and TLR-independent manner [12–14]. Furthermore, purified and synthetic versions of α-glucuronosylceramide (GSL-1; Fig. 2) and to a lesser extent α-galacturonosylceramide (GSL-1′), both found in *Sphingomonas* spp., were able to stimulate murine and human iNKT cells in a TLR- and IL-12-independent manner. CD1d-tetramers loaded with GSL-1 stained human and murine iNKT cells, providing rigorous proof that indeed these lipids are recognized directly by the iNKT cell TCR. In addition to the monoglycosylceramide GSL-1, members of the *Sphingomonas* family of bacteria also express GSL-3 and GSL-4, both containing an additional glucosamine along with one or two additional sugars, respectively [14–16]. However, synthetic versions of these oligoglycosylceramides showed limited or no stimulatory capacity for iNKT cells and APCs were unable to process these lipids to the stimulatory monoglycosylceramide GSL-1. Thus, *Sphingomonas* spp. bacteria express α-linked glycosylceramides that are recognized by the iNKT cell TCR and are capable of activating iNKT cells in the absence of TLR-signaling or -stimulation by IL-12, both hallmarks of the indirect, cytokine-driven pathway of iNKT cell activation (discussed below). During infection with *Sphingomonas* spp. bacteria in mice, iNKT cells appear to control both bacterial clearance and the septic shock response. While members of the *Sphingomonadaceae* family generally display low pathogenicity and rarely cause infections in humans, carriage or infection with *Novosphingobium aromaticivorans* appears to be involved in the pathogenesis of primary biliary cirrhosis, an autoimmune disorder affecting the liver [17].

Microbial glycolipids that are directly recognized by iNKT cells have also been isolated from the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease. Mice deficient in iNKT cells have impaired pathogen clearance and are more prone to develop inflammatory joint and cardiac disease following infection with *B. burgdorferi* [18–20]. Infection of mice with this pathogen through tick bites or transfer of BM-DCs pulsed with *B. burgdorferi* cell lysate resulted in activation of iNKT cells [21]. One of the dominant lipids expressed by *B. burgdorferi* is BbGL-II, a diacylglycerol lipid containing an α-linked galactose (Fig. 2). Purified and synthetic forms of BbGL-II incubated with plate-bound CD1d molecules activated iNKT cells, and BbGl-II loaded CD1d tetramers stained a subpopulation of murine iNKT cells. Both of these features rigorously confirm the direct antigenic nature of these lipids for the iNKT cell TCR. iNKT cell activation with BbGL-II *in vitro* did not require TLR-mediated signaling. Interestingly, human and murine iNKT cells differentially responded to variants of BbGL-II that differ in length and number of double bonds of their alkyl chains, suggesting that the alkyl chains of this lipid contribute to its antigenicity.

Together, these results demonstrate that some bacteria express microbial lipids that are presented by CD1d molecules, leading to TCR-mediated activation of iNKT cells in the absence of TLR- and cytokine-mediated stimulation (Fig. 1, left panel). Additional CD1d-presented microbial lipid antigens are likely present in *Plasmodium falciparum*, *Trypanosoma* spp., *Leishmania* spp., *Ehrlichia* spp., *Streptococcus pneumoniae*, and *Helicobacter pylori* as well as in *M. bovis* BCG [12,22–25]. Rigorous criteria for defining CD1d-presented microbial lipid antigens are emerging and include: iNKT cell activation in the absence of IL-12-, IL-18-, or type I IFN-mediated stimulation following TLR-mediated APC-activation, iNKT cell activation in APC-free plate binding assays using recombinant CD1d proteins, staining of iNKT cells with antigen-loaded CD1d tetramers, as well as generation of synthetic versions of the proposed microbial lipid antigen structures. The unifying structural feature of microbial iNKT cell antigens found in *Sphingomonas* spp. and *B. burgdorferi* is a hexose sugar (glucose or galactose) in an α-anomeric linkage with either a glycosphingolipid or a diacylglycerol (Fig. 2). Interestingly, recognition of these α-linked microbial glycolipids by the iNKT cell TCR appears to be mediated predominantly by a germline-encoded region of the invariant TCRα chain, while regions in the TCRβ chain appear to modulate the overall affinity of the iNKT cell TCR for CD1d/lipid complexes [26–30]. This mode of recognition of microbial products by iNKT cells bears similarity to the recognition of microbial products by pattern-recognition receptors such as TLRs [28,31]. In fact, it has been proposed that, in the case of the LPS-negative Gram(−) *Sphingomonas* spp. bacteria, CD1d-mediated recognition of microbial glycosphingolipids by iNKT cells may be an innate immune mechanism allowing detection of microorganisms that lack TLR ligands [12,13]. The recently described microbial antigens and the numerous αGalCer analogs that have been shown to activate iNKT cells reveal that the TCR of a single iNKT cell recognizes several structurally diverse exogenous antigens, which is in stark contrast to the high degree of specificity found among MHC class I- and II-restricted T cells. In addition to their apparent promiscuity, individual iNKT cell TCRs may also have non-overlapping, exclusive specificities for distinct microbial lipids, as has been suggested by the private specificity of a human iNKT cell clone for a mammalian phospholipid [32].

3. Indirect, cytokine- and self-antigen-driven pathway of iNKT cell activation during infection

In contrast to the direct recognition of microbial lipid antigens by iNKT cells, a second pathway has emerged that can lead to overt iNKT cell activation without the need for TCR-mediated recognition of microbial lipids. The first evidence for what is now commonly referred to as the “indirect pathway of iNKT cell activation” came from studies using human iNKT cell clones in co-culture with monocyte-derived dendritic cells (DC) and heat-killed *S. typhimurium* or *S. aureus* bacteria resulting in IFN-γ secretion by iNKT cells [33]. This iNKT cell activation was CD1d-dependent but did not appear to require CD1d-presented microbial lipid antigens from *S. typhimurium* or *S. aureus* bacteria. In fact, purified LPS alone was capable of fully activating iNKT cells in a CD1d-dependent manner in the presence of DCs. This led to the discovery that IL-12, a pro-inflammatory cytokine released by DCs after exposure to bacterial products such as LPS, is capable of stimulating iNKT cells in combination with a weak TCR-mediated signal received by recognition of CD1d-presented self-antigens (Fig. 1, middle panel). *In vivo* activation of iNKT cells during *S. typhimurium* infection occurred rapidly and could be blocked significantly with antibodies against CD1d or IL-12, confirming the requirement for IL-12 stimulation and CD1d recognition during infection. A role for TLR-mediated signaling in this indirect, cytokine-driven pathway of iNKT cell acti-

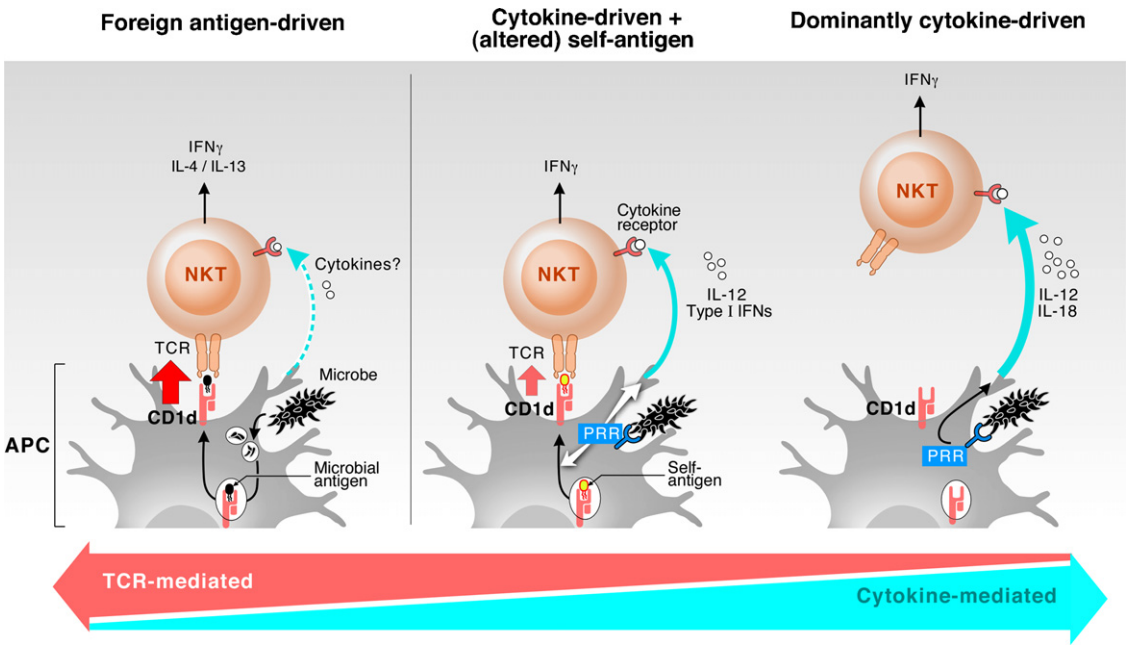

Fig. 1. Different pathways leading to iNKT cell activation during infection. Following stimulation with microbial products or following infection, iNKT cells are activated by a combination of cytokine- and TCR-mediated signals. *Left panel*: Foreign antigen-driven: microbial lipids that can be loaded into CD1d molecules leading to iNKT cell stimulation have been isolated from *Sphingomonas* spp. and *B. burgdorferi*. *Middle panel*: Cytokine- and self-antigen-driven: in response to TLR2, TLR3, TLR4, TLR7 and TLR 9 agonists and during infection with *S. typhimurium*, IFN-γ-secretion by iNKT cells is induced by a combination of pro-inflammatory cytokines (IL-12, IL-18 or type I IFNs) released by DCs and stimulation of the iNKT cell TCR by CD1d-presented self-antigens. Following TLR-stimulation, alterations in the biosynthetic pathways of glycosphingolipids have been described that lead to display of altered, more stimulatory self-lipids by CD1d molecules. *Right panel*: Dominantly cytokine-driven: in response to *E. coli* LPS or viral infection, production of pro-inflammatory cytokines such as IL-12 and IL-18 by DCs results in IFN-γ-release by iNKT cells with a reduced need for additional TCR-mediated stimulation.

vation was confirmed by studies using DCs deficient in the adaptor molecule MyD88, which lacked the ability to stimulate iNKT cells in the presence of heat-killed *S. typhimurium* [12]. Several other studies have confirmed that the indirect, cytokine-mediated pathway of iNKT cell activation is operational during microbial infection. For example, during infection with the parasite *Trypanosoma cruzi* iNKT cell activation could be inhibited *in vivo* with blocking antibodies against CD1d and was dependent on expression of IL-12,

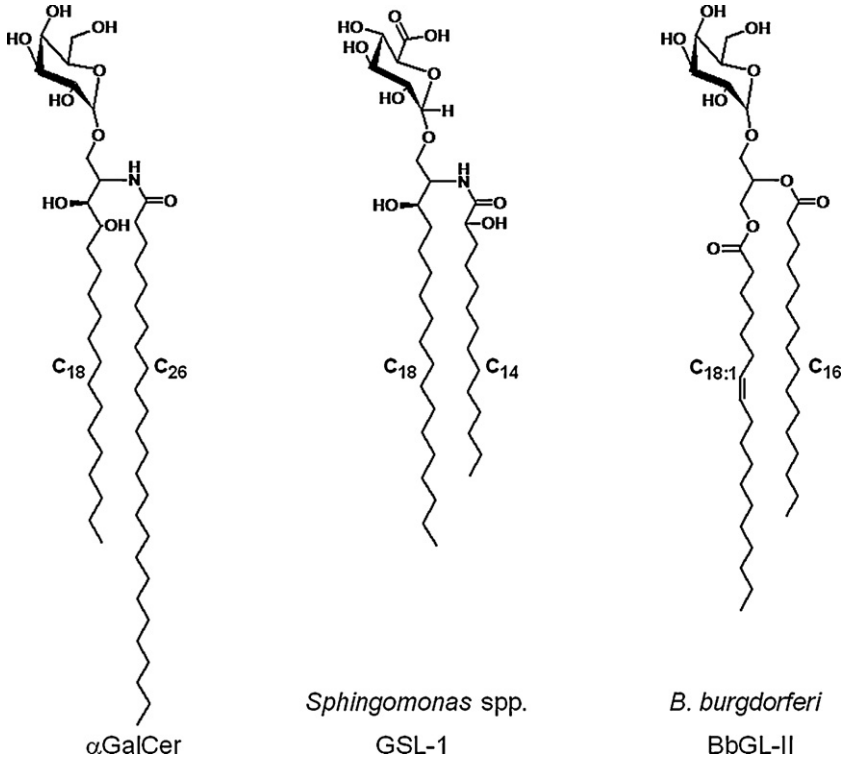

Fig. 2. Structures of CD1d-presented microbial lipid antigens. Antigens recognized by iNKT cells include the α-glucuronosylceramide GSL-1 from *Sphingomonas* spp. and the diacylglycerol BbGL-II isolated from *B. burgdorferi*. The structure of the synthetic αGalCer antigen is shown for comparison. The shared structural feature of these iNKT cell antigens is the alpha linkage of the glycan headgroup.

but not MyD88 [34]. This suggests that iNKT cell activation during *T. cruzi* infection requires both TCR- and IL-12-mediated stimulation. However, the release of IL-12 during this infection appears to be stimulated in a TLR-independent manner and the receptors and signaling molecules involved in its stimulation remain to be determined. A lipopeptidophosphoglycan isolated from *Entamoeba histolytica* (EhLPPG) stimulated iNKT cells in an IL-12- and TLR-dependent manner [35]. Interestingly, EhLPPG was taken up by DCs and treatment of DCs with bafilomycin A1, an inhibitor of endocytosis, abrogated the ability of this lipid to stimulate iNKT cells. In contrast, iNKT cell stimulation by the TLR agonists Pam3Cys or LPS was not inhibited by treatment with bafilomycin A1. This suggests that EhLPPG, but not Pam3Cys or LPS, requires uptake and intracellular processing in order to stimulate iNKT cells. The role for uptake and endocytosis of EhLPPG in this example of TLR- and cytokine-mediated activation of iNKT cells remains to be determined. A study using eggs from the parasite *Schistosoma mansoni* to stimulate murine liver mononuclear cells (LMNCs) as responders suggested that IFN-γ and IL-4 secretion by iNKT cells was dependent on both CD1d expression and self-antigen presentation by DCs rather than the presentation of microbial lipids [36]. However, LMNCs from IL-12- or MyD88-deficient mice responded similar to wild type LMNCs, suggesting that iNKT cell stimulation with *S. mansoni* lipids is dependent on the recognition of CD1d-presented self-lipids but independent of IL-12 and TLR-signaling. Another example of indirect, cytokine-driven iNKT cell activation in response to infection comes from studies investigating the iNKT cell response to *Mycobacterium tuberculosis* infection. Macrophages infected with *M. tuberculosis* stimulated iNKT cells in a CD1d- and IL-12/IL-18-dependent manner and iNKT cell activation led to reduced numbers of bacteria in iNKT cell co-cultures with infected macrophages, suggesting that iNKT cell activation again is dependent on cytokine-driven activation [37]. Interestingly, as discussed above, PIM4, isolated from *M. bovis* BCG, has been suggested to be a CD1d-presented microbial antigen capable of direct iNKT cell activation. This lipid is expected to be present also in *M. tuberculosis* and if indeed confirmed as an iNKT cell antigen, it is possible that even during infection with a pathogen that expresses an iNKT cell antigen, TLR- and IL-12/18-mediated iNKT cell activation may dominate over the stimulation received from the microbial antigen.

In summary, these studies demonstrate that iNKT cells can use a unique mechanism of activation in response to microbial infection that does not require TCR-mediated cognate recognition of CD1d-presented microbial lipid antigens. Instead, weak responses to CD1d-presented self-antigens are amplified by inflammatory cytokines, such as IL-12, secreted by DCs in response to microbial products, resulting in potent iNKT cell activation (Fig. 1, middle panel).

3.1. Infection-induced expression of altered self-lipids

Understanding the nature of CD1d-presented self-lipids that are critical for iNKT cell development and function is a major focus in the field of CD1/iNKT cell biology [25].

CD1d molecules are thought to capture self-lipids in the endoplasmic reticulum, along the secretory pathway to the cell surface, or in the recycling pathway [2]. Self-lipids bound by CD1d molecules include phospholipids (PC, PE, PI, GPI, cardiolipin), lysophospholipids, plasmalogens, sphingomyelin, the ganglioside GM3, and the isogloboside iGb3 [38–44]. Thus, a surprising diversity of cellular lipids is bound by CD1d molecules in different intracellular compartments. However, it is still unclear which of these lipids actually stimulate iNKT cells under physiologic conditions and which contribute to the activation of iNKT cells in the context of microbial infection.

Inflammation and infection are known to alter the expression of enzymes of lipid metabolism and thus the composition of cellular lipids [45]. Two recent studies have elegantly addressed the intriguing possibility that lipids bound to CD1d molecules are altered following stimulation with TLR agonists or during infection, thus contributing to the activation of iNKT cells in response to microbes.

Human iNKT cell lines co-cultured with monocyte-derived DCs in the presence of the TLR agonists Pam3Cys (TLR2), poly:IC (TLR 3), LPS (TLR4), flagellin (TLR5), R848 or ssRNA40 (both TLR8) resulted in IFN-γ secretion, suggesting that a broad range of TLR stimuli can initiate the indirect, cytokine-driven, pathway of iNKT cell activation [46]. Interestingly, R848-induced activation of iNKT cells was inhibited when DCs were matured in the presence of *N*-butyldeoxygalactonojirimycin (*N*B-DGJ), an inhibitor of glycosphingolipid synthesis. In addition, expression of several enzymes involved in the biosynthesis of glycosphingolipids of the ganglio and globo series was higher in R848-matured, compared with immature DCs. These findings suggest that enhanced or altered synthesis of glycosphingolipids contributed to iNKT cell activation following TLR8 stimulation. Indeed, lipids extracted from APCs stimulated with R848 induced slightly stronger iNKT cell activation in the presence of suboptimal amounts of IL-12p70 than lipids extracted from un-stimulated APCs. Furthermore, higher levels of soluble Vα24Jα18/Vβ11 TCR hetero-dimer bound to CD1d-expressing APCs following stimulation with R848 or LPS, while CD1d surface levels were unchanged. Thus, TLR stimulation induces changes in the lipid composition of APCs and specific up-regulation of CD1d-presented ligands appears to contribute to iNKT cell activation in response to microbial products.

In a second study, the TLR agonists LPS (TLR4), R848 (TLR8), or CpG (TLR9) stimulated secretion of IFN-γ, but not IL-4, by murine iNKT cells in co-culture with bone marrow-derived DCs [47]. iNKT cell activation with CpG oligonucleotides was CD1d- and IFN-α receptor (IFNAR1)-dependent and IL-12-independent, suggesting that the TLR-induced iNKT cell activation in this system requires a TCR-mediated signal and is amplified by type I IFNs. CpG-induced iNKT cell activation was reduced when DCs were stimulated with CpG in the presence of the glycosphingolipid synthesis inhibitor *N*B-DGJ, and charged lipids isolated from CpG-stimulated DCs were able to activate iNKT cells in the presence of suboptimal levels of recombinant IFN-β. Furthermore, similar to findings described above, several enzymes involved in the synthesis of glycosphingolipids of the ganglio, (iso)globo, and (neo)lacto series were up-regulated following CpG stimulation.

Together, these studies suggest that stimulation of DCs with TLR agonists results in altered glycosphingolipid synthesis and exchange of weakly stimulatory CD1d-presented self-ligands with more stimulatory ligands, thus contributing to iNKT cell activation (Fig. 1, middle panel). However, the nature of the lipids preferentially loaded into CD1d molecules following TLR stimulation or infection remains to be determined. Increased synthesis of endogenous GSLs following infection or exposure to microbial products has also been shown to result in overt activation of CD1a- and CD1b-restricted, sulfatide- and GM1-specific T cells, respectively [48]. In a recent report, lyso-phosphatidylcholine, known to be generated from PC during inflammatory conditions, has been shown to stimulate human iNKT cells, providing a further example of how altered self-lipids may contribute to iNKT cell activation during inflammatory responses [49].

3.2. Modulation of CD1d expression during infection

The expression of MHC class I and class II molecules on the surface of APCs is regulated following activation by inflammatory stimuli or infection and contributes to T cell activation. Similarly, several reports show that the expression of CD1d molecules on

the surface of APCs is modulated following stimulation with TLR agonists or infection, resulting in altered iNKT cell activation.

CD1d expression levels were increased on macrophages, DCs, and, to a lesser extent, on B cells during the course of *L. monocytogenes* infection *in vivo* [50,51]. *In vitro* infection of bone marrow-derived DCs with *L. monocytogenes* resulted in increased surface expression of CD1d in an IFN-β-dependent manner [51]. Recombinant IFN-β was sufficient to induce increased *de novo* CD1d synthesis in APCs but had no effect on intracellular distribution, internalization or recycling of CD1d molecules. Thus, increased CD1d expression on APCs following *L. monocytogenes* infection is IFN-β-dependent and the result of increased protein synthesis rather than re-distribution of CD1d molecules. Following *in vitro* infection of murine macrophages with *M. tuberculosis*, surface expression of CD1d was up-regulated when additional recombinant IFN-γ was provided, suggesting that two signals, one provided by IFN-γ and a second provided by microbial products, are required for this effect [52]. The second signal can be provided by the TLR2 agonist Pam3Cys, the TLR4 agonist LPS or the pro-inflammatory cytokine TNF-α. During *M. tuberculosis* infection in mice *in vivo* CD1d surface expression increased on macrophages in an IFN-γ-dependent manner. Thus, the interplay between cytokines and microbial signals appears to regulate CD1d expression on macrophages during *M. tuberculosis* infection. TLR or cytokine stimulation has also been shown to modulate CD1d expression on human DCs. Increased CD1d mRNA and protein levels were observed in human monocyte-derived DCs following stimulation with UV-inactivated herpes simplex virus 1 (HSV1), IFN-α, IFN-β, or the TLR7 agonist imiquimod [53]. Thus, viral danger signals and type I IFNs induce enhanced CD1d expression levels on human DCs through enhancing CD1d mRNA transcription and CD1d protein translation. Additional examples suggest that in response to microbial products or during infection, CD1d levels can be increased on APCs. For example, following infection of murine BM-DCs *in vitro* with the Gram(−) bacterium *S. typhimurium* or stimulation with LPS, CD1d expression levels were up-regulated [54]. However, no change in CD1d expression levels was observed on DCs following *in vivo* infection with *S. typhimurium*. Increased expression of CD1d, partially dependent on TNFα expression, was observed on endothelial cells following infection with Coxsackievirus B3, an infection that induces myocarditis [55]. Together, these studies document that CD1d expression levels are increased on APCs following exposure to microbial products and during infection.

Increased CD1d expression levels on APCs have been shown to result in enhanced activation of iNKT cells. For example, IFN-β-stimulated BM-DC and BM-DC from transgenic mice overexpressing CD1d under the control of a MHC class I promoter induced higher levels of IFN-γ and IL-4 secretion from iNKT cells in the absence of exogenous antigen [51]. Furthermore, increased cell surface expression levels of CD1d on macrophages and CD1d-transfected RMA-S cells selected for varying CD1d expression levels correlated with increased activation of iNKT cell hybridomas and primary iNKT cells, independently of whether the iNKT cell reactivity was to self- or foreign lipid antigens [52]. Thus, increased CD1d expression levels on APCs result in enhanced stimulation of iNKT cells in response to endogenous and exogenous antigens.

However, not all studies have observed an increase in CD1d expression on APCs following exposure to microbial products. For examples, exposure of human monocyte-derived DCs to mycobacterial cell wall products decreased both CD1d mRNA and protein levels, while increasing the expression levels of group 1 CD1 molecules [56]. Furthermore, several reports have shown that interference with the expression of CD1 molecules as well as the presentation of endogenous and exogenous lipids by CD1d molecules enable pathogens to evade recognition by CD1d-restricted T cells. For example, infection with human immunodeficiency virus 1 (HIV-1), HSV-1, Kaposi sarcoma-associated herpes virus (KSHV), varicella zoster virus (VZV), vesicular stomatitis virus (VSV) and *Chlamydia trachomatis* appear to interfere with CD1d antigen presentation on APCs by enhancing CD1d internalization, blocking delivery of CD1d to the cell surface, destabilization of CD1d and interference with the MAPK signaling pathway (reviewed in [2]).

Together, these studies suggest that modulation of CD1d expression on APCs following stimulation with microbial products and during infection can contribute to the regulation of iNKT cell activation by enhancing or reducing the TCR-mediated stimulation of iNKT cells by CD1d-presented ligands (Fig. 1, middle panel).

3.3. Dominantly cytokine-driven iNKT cell activation during infection

iNKT cells also were recognized for their ability to secrete IFN-γ in response to recombinant innate cytokines, such as IL-12 and IL-18, independently of additional TCR stimulation [57,58]. Indeed, several recent reports have demonstrated that in response to microbial products or infections that generate significant amounts of potent pro-inflammatory, IFN-γ-inducing cytokines, iNKT cell activation is predominantly cytokine-driven, reducing the role of CD1d/TCR-mediated stimulation.

Following i.v. injection of *E. coli* LPS in C57BL/6 mice, iNKT cells in spleen and liver expressed IFN-γ, but no IL-4 [59]. This response was significantly reduced in IL-12p40-deficient mice and completely absent in IL-18-deficient mice, suggesting that both IL-12 and IL-18 play an important role in the iNKT cell response to *E. coli* LPS. A strong dependence on IL-12 and IL-18 was also observed for the iNKT cell response to *E. coli* LPS *in vitro*. However, this response was not significantly reduced using CD1d-deficient APCs. Together, these results suggest that in response to *E. coli* LPS, iNKT cell activation is dominantly cytokine-driven and has a reduced requirement for TCR-mediated recognition of CD1d-presented self-antigens. In contrast to the finding that stimulation of DCs with the TLR9 agonist CpG results in iNKT cell activation through combined stimulation by altered CD1d-presented self-antigens and IFN-β (Ref. [47], discussed above), two recent studies have suggested that the iNKT cell response to CpG stimulation is dependent on IL-12, but largely independent of CD1d recognition [60,61]. The reasons for this discrepancy remain to be determined but may include subtle differences in the experimental systems and protocols used.

CpG oligonucleotides are considered “viral danger signals” and recent studies have examined iNKT cell activation in response to viral infection. Infection of BM-DCs with murine CMV (mCMV) *in vitro* resulted in IFN-γ-, but not IL-4-secretion by iNKT cells. This iNKT cell response was completely TLR9- and IL12p40-dependent, and only partially IL-18- and CD1d-dependent [61]. During mCMV infection *in vivo*, iNKT cells were activated as soon as 12 h following infection, as determined by increased expression of CD25 and intracellular IFN-γ. This *in vivo* response was strongly decreased in IL-12p40- and TLR9-deficient mice, and only partially decreased in IL-18-deficient mice [61,62]. Treatment of mice with anti-CD1d mAb before and during mCMV infection resulted in slightly decreased IFN-γ production by iNKT cells. In addition, adoptive transfer of wild-type iNKT cells into CD1d-deficient or wild-type mice resulted in comparable iNKT cell activation following mCMV infection [62]. Thus, iNKT cell activation during mCMV infection is dominantly driven by the pro-inflammatory cytokine IL-12, with a reduced requirement for CD1d/TCR-mediated stimulation.

In summary, these studies clearly document that iNKT cell activation during infection does not require the recognition of microbial CD1d-presented lipid antigens. Instead, pro-inflammatory cytokines released by APCs following stimulation with microbial

products, together with changes in the display of CD1d-presented self-antigens from weak to more stimulatory ligands, and increases in CD1d surface expression during certain infections, all can contribute in a concerted manner to full iNKT cell activation in response to microbial infection (Fig. 1, middle panel). Under circumstances where very potent pro-inflammatory, IFN-γ-inducing cytokines are secreted by APCs in response to microbial products, overt iNKT cell activation is dominantly cytokine-driven and the role for a CD1d/TCR-mediated signal appears reduced (Fig. 1, right panel). However, it has been shown recently that in order for CD1d-mediated self-reactivity to potentiate the ability of iNKT cells to respond to co-stimulatory cytokines, the concurrent recognition of CD1d is not required. Instead, self-reactive stimulation appears to "prime" iNKT cells for subsequent activation by co-stimulatory cytokines such as IL-12 [63].

The activation of iNKT cells in response to stimulation with TLR agonists has raised the intriguing possibility that TLRs may be expressed by iNKT cells and thus responsible, or at least involved, in the activation of iNKT cells during infection [64,65]. However, several studies have not been able to confirm the expression of TLR4 or TLR9 on iNKT cells or a direct activation of iNKT cells by LPS or CpG [59,66].

4. Summary and conclusions

Recent progress has shed light on the question how iNKT cells, with their restricted TCR diversity, can respond rapidly to a wide range of bacterial, viral, protozoan, and fungal pathogens. Emerging evidence suggests that iNKT cells are activated during infection either by directly recognizing microbial lipid antigens with their TCR, or, in the absence of such antigens, by a cytokine-driven mechanism that involves the recognition of CD1d-presented self-antigens (Fig. 1).

For the direct, antigen-driven mode of iNKT cell activation, there are now several examples of bacteria expressing microbial lipids that can be recognized by the iNKT cell TCR after being loaded into CD1d molecules. However, it remains to be determined to what extent microbial antigenic lipids are available for presentation by CD1d molecules to iNKT cells during infection and how such lipids contribute uniquely to the protective immune response against these infectious agents. Purified and synthetic version of the microbial lipid antigens recognized by iNKT cells are sufficient to overtly stimulate IFN-γ secretion. However, cytokine-mediated stimulation may play an important role for iNKT cell activation during the course of infection with pathogens that express both TLR ligands and CD1d-presented lipid antigens. For example, the spirochete *B. burgdorferi* has been shown to synthesize both antigenic diacylglycerol lipids that can be recognized by iNKT cells and lipoproteins that stimulate TLR2 and induce the release of pro-inflammatory cytokines [67]. Since it has been shown that iNKT cell responses to suboptimal concentrations of the model antigen αGalCer can be amplified by addition of suboptimal concentrations of recombinant IL-12 or following CpG-mediated stimulation [66,68], it appears likely that during infection, cytokine-mediated stimulation of iNKT cells occurs in addition to their stimulation by cognate microbial lipid antigens (Fig. 1, left panel).

In contrast to the direct recognition of microbial lipids, in the indirect mechanism of activation, iNKT cell stimulation is achieved by a combination of innate cytokines, such as IL-12, IL-18, or type I IFNs, secreted by APCs in response to microbial products, together with a weak TCR-mediated signal provided by recognition of constitutive or altered self-lipid antigens (Fig. 1, middle panel). Display of altered, more stimulatory lipids by CD1d molecules in response to microbial products and infection contributing to the activation of iNKT cells provides an elegant way of sensing changes in the cellular lipid metabolism during infection. However, the nature of infection-induced, altered CD1d-presented self-antigens capable of stimulating iNKT cells remains to be determined. The recognition of "self" or "altered self" by the semi-invariant iNKT cell TCR in the context of microbial infection provides a critical TCR-mediated signal for iNKT cell activation in combination with cytokine-mediated stimulation, allowing iNKT cell activation in response to a wide variety of bacterial, protozoan and viral pathogens. This is particularly important for the recognition of viral pathogens, since viruses are not expected to contain "microbe-specific" unique lipids, and for pathogens that do not express cognate iNKT cell lipid antigens. The indirect, cytokine-driven activation of iNKT cells can be modulated by increased expression of CD1d molecules, contributing to stronger TCR signaling. In response to strong TLR-mediated stimulation and in particular during viral infection, DCs can be stimulated to secrete a potent combination of synergistic, pro-inflammatory cytokines that provide very strong cytokine-mediated stimulation for iNKT cells (Fig. 1, right panel). This can reduce or abrogate the need for TCR-mediated stimulation in order to achieve overt iNKT cell activation and may be particularly helpful in the immune response to pathogens that down-regulate or interfere with CD1d-mediated antigen presentation.

Overall, the indirect mechanism of iNKT cell activation bears resemblance to the cytokine-mediated activation of NK cells and γδ T cells in response to microbial products and during infection [69–72]. In addition, antigen-experienced CD8 T cells and CD4 T cells are known to acquire the ability to respond to innate stimuli that induce IFN-γ production in the absence of cognate antigen stimulation and have been shown to play a beneficial role in controlling infections [73,74]. Thus, as a result of exogenous antigen-independent, innate cytokine-driven activation, iNKT cells, along with NK cells, γδ T cells and antigen-experienced CD8 and CD4 T cells, may contribute to the control of a wide range of infections during a phase immediately following the innate immune response, but before the development of the adaptive immune response [33,75]. This provides the evolving immune response with T cell effector functions early during the course of infection that otherwise would be available only after the expansion and differentiation of MHC-restricted T cells. In addition to their role in bridging the temporal gap between the innate and adaptive immune response, iNKT cells have emerged as critical amplifiers of the early immune response to microbial products and infection. This has been demonstrated by several recent studies showing a dependence of the NK cell response on the preceding activation of iNKT cells, following stimulation with microbial products and during infection [59,60,62].

In summary, dependent on the nature of the pathogen and its associated molecular patterns, either TCR-mediated stimulation, cytokine-mediated stimulation, or a combination of cytokine- and TCR-mediated stimulation result in iNKT cell activation during infection (Fig. 1). The mode of iNKT cell activation in the absence of foreign antigen recognition during a primary response to infection is unique for cells bearing TCRs and underscores that not only the function but also the activation mechanism of iNKT cells is innate-like, and distinct from adaptive T cells. Interestingly, the different modes of activation of iNKT cells appear to result in differential effector functions. For example, IL-12-driven activation of iNKT cells appears to stimulate cytotoxicity more strongly than stimulation with antigen [76]. Stimulation with IL-12, IL-18 or type I INFs appears to result predominantly in secretion of IFN-γ, with no or little IL-4. In contrast, the stimulation of iNKT cells with cognate microbial antigens results in secretion of both, IFN-γ and IL-4. Thus, dependent on the microbial pathogen and the resulting mechanism of activation, the ensuing iNKT cells response may be tuned differently. Understanding the role of pro-inflammatory cytokines as well as of self, altered self and microbial lipids in the process of activating CD1d-restricted iNKT cells provides critical information on

how these T cells are capable to contribute to the immune defense in response to infection. Exploiting this knowledge promises new approaches to adjuvant and vaccine strategies.

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