Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection

Manfred Brigl,[1,2] Raju V.V. Tatituri,[2] Gerald F.M. Watts,[2] Veemal Bhowruth,[3] Elizabeth A. Leadbetter,[2] Nathaniel Barton,[2] Nadia R. Cohen,[2] Fong-Fu Hsu,[4] Gurdyal S. Besra,[3] and Michael B. Brenner[2]

[1]Department of Pathology and [2]Department of Medicine, Division of Rheumatology, Immunology, and Allergy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115
[3]School of Biosciences, University of Birmingham, Birmingham B15 2TT, England, UK
[4]Division of Endocrinology, Metabolism and Lipid Research, Washington University, St. Louis, MO 63110

Invariant natural killer T cells (iNKT cells) are critical for host defense against a variety of microbial pathogens. However, the central question of how iNKT cells are activated by microbes has not been fully explained. The example of adaptive MHC-restricted T cells, studies using synthetic pharmacological α-galactosylceramides, and the recent discovery of microbial iNKT cell ligands have all suggested that recognition of foreign lipid antigens is the main driver for iNKT cell activation during infection. However, when we compared the role of microbial antigens versus innate cytokine-driven mechanisms, we found that iNKT cell interferon-γ production after in vitro stimulation or infection with diverse bacteria overwhelmingly depended on toll-like receptor–driven IL-12. Importantly, activation of iNKT cells in vivo during infection with *Sphingomonas yanoikuyae* or *Streptococcus pneumoniae*, pathogens which are known to express iNKT cell antigens and which require iNKT cells for effective protection, also predominantly depended on IL-12. Constitutive expression of high levels of IL-12 receptor by iNKT cells enabled instant IL-12–induced STAT4 activation, demonstrating that among T cells, iNKT cells are uniquely equipped for immediate, cytokine-driven activation. These findings reveal that innate and cytokine-driven signals, rather than cognate microbial antigen, dominate in iNKT cell activation during microbial infections.

CORRESPONDENCE
Michael B. Brenner:
mbrenner@rics.bwh.harvard.edu
OR
M. Brigl:
mbrigl@rics.bwh.harvard.edu

Abbreviations used: GalCer, galactosylceramide; Gal-GlcDAG, galactosyl GlcDAG; GlcDAG, glucosyldiacylglycerol; GSL, glycosphingolipid; iNKT cell, invariant NK T cell; MFI, mean fluorescence intensity; TLR, toll-like receptor.

Invariant NK T cells (iNKT cells) recognize microbial and endogenous cellular lipid antigens presented by CD1d molecules (Brigl and Brenner, 2004; Kronenberg, 2005; Bendelac et al., 2007). iNKT cells express an invariant TCR-α chain (Vα14Jα18 in mice and Vα24Jα18 in humans) paired with a limited set of TCR-β chains, display surface receptors typically found on NK cells, and have a memory/effector phenotype in the absence of prior stimulation (Godfrey et al., 2004). iNKT cells constitutively express mRNA, but not protein, for IFN-γ, poising them for rapid effector function (Stetson et al., 2003). Together, these features distinguish iNKT cells from MHC-restricted T cells and suggest distinct modalities of activation.

A growing body of evidence documents a critical role for iNKT cells during bacterial, viral, fungal, and protozoan infections (Tupin et al., 2007; Cohen et al., 2009). The protective, and in some instances detrimental, functions of iNKT cells during infection are often the result of their ability to rapidly produce copious amounts of IFN-γ and to contribute to the recruitment and activation of other cell types, including neutrophils, macrophages, DCs, NK cells, and B cells. These properties enable iNKT cells to orchestrate and amplify the protective immune response to infection (Brigl and Brenner, 2004; Tupin et al., 2007; Cohen et al., 2009). Yet how CD1d-restricted iNKT cells, with their limited TCR diversity, become activated rapidly

Elizabeth A. Leadbetter's present address is Trudeau Institute, Saranac Lake, NY 12983.

R.V.V. Tatituri and G.F.M. Watts contributed equally to this paper.

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 208 No. 6 1163-1177
www.jem.org/cgi/doi/10.1084/jem.20102555

The Journal of Experimental Medicine

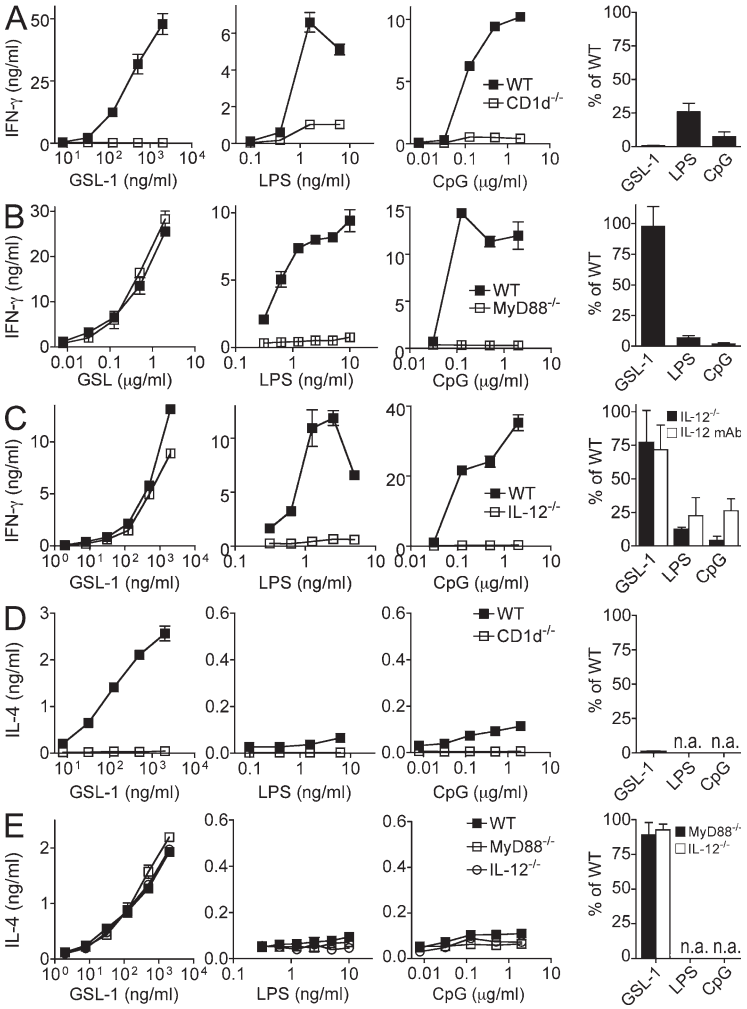

Figure 1. Antigen- versus cytokine-driven pathways of iNKT cell activation. (A–C) iNKT cell lines were cultured with WT (filled squares) and CD1d-deficient (A), MyD88-deficient (B), or IL-12p35-deficient (C; all open squares) BM-derived DCs and stimulated with various concentrations of LPS, CpG, or GSL-1 for 16–24 h. Cytokine concentrations in culture supernatants were measured by ELISA. Data are presented as means of duplicate values ± SD and are representative of at least three independent experiments. Bar graphs show percent inhibition of IFN-γ secretion in culture supernatants comparing CD1d-deficient (A), MyD88-deficient (B), or IL-12p35-deficient (C) DCs (filled bars) to WT DCs after stimulation with 2 ng/ml LPS, 2 μg/ml CpG, or 10 μg/ml GSL-1, and data are summarized from four independent experiments (mean ± SD). Open bars in C represent the percentage of IFN-γ secretion in the presence of 10 μg/ml of blocking anti–IL-12 antibodies. Data are summarized from two independent experiments. No significant inhibition was observed with control antibodies (not depicted). (D and E) Experiments showing IL-4 production in response to GSL-1, LPS, or CpG using CD1d- (D) and MyD88- and IL-12–deficient (E) BM DCs were performed as in A–C. IL-4 concentrations in culture supernatants were measured by ELISA. n.a. indicates not applicable because IL-4 levels with WT DCs were very low. Data are presented as means of duplicate values ± SD and are representative of at least three independent experiments.

in response to vastly diverse microbial infections remains incompletely understood.

Two distinct pathways have been described for iNKT cell activation during microbial infection. The microbial antigen-driven pathway involves direct recognition of CD1d-presented microbial lipid antigens by iNKT cells. Glycosphingolipids (GSLs) found in *Sphingomonas* spp. (Kinjo et al., 2005; Mattner et al., 2005; Sriram et al., 2005) and diacylglycerols isolated from both *Borrelia burgdorferi* (Kinjo et al., 2006) and *Streptococcus pneumoniae* (Burrows et al., 2009) can be presented by CD1d molecules, and these microbial lipid antigens have been proposed to drive iNKT cell activation in a TCR-dependent manner during infection (Kinjo et al., 2005, 2006; Mattner et al., 2005). After exposure to these microbial antigens, iNKT cells produce both IFN-γ and IL-4 within hours (Kinjo et al., 2005, 2006; Mattner et al., 2005). The recognition of microbial glycosylceramides by iNKT cells has been proposed to fill a gap in the innate recognition of Gram-negative LPS-negative α-proteobacteria such as *Sphingomonas* spp. and *Ehrlichia* spp. (Kinjo et al., 2005; Mattner et al., 2005). The production of IFN-γ by iNKT cells in response to antigen stimulation does not require IL-12 signaling; however, IL-12 is known to play a critical role in the trans-activation of NK cells and the systemic release of IFN-γ after iNKT cell stimulation (Kitamura et al., 1999; Kawakami et al., 2001; Matsuda et al., 2003).

In contrast to the TCR-mediated recognition of microbial lipid antigens, iNKT cells can be activated fully in response to microbial products by an innate cytokine- and self-antigen–driven pathway. In this scenario, iNKT cell activation results from combined stimulation with a weak TCR-mediated signal from recognition of endogenous CD1d-presented lipids, together with cytokine-mediated co-stimulation by IL-12, released by DCs after toll-like receptor (TLR)–mediated activation (Brigl et al., 2003; Mattner et al., 2005; Nagarajan and Kronenberg, 2007). iNKT cell activation after stimulation of DCs with TLR agonists can be modulated by alterations in CD1d-presented self-lipids and changes in CD1d expression levels (Sköld et al., 2005; Raghuraman et al., 2006; Paget et al., 2007; Salio et al., 2007). In some cases, such as stimulation with LPS from *Escherichia coli* or during viral infection, iNKT cell activation can be so dominantly driven by IL-12 and IL-18 that very little or no TCR-mediated stimulation by CD1d-presented self-lipids is needed (Nagarajan and Kronenberg, 2007; Tyznik et al., 2008; Wesley et al., 2008). This innate cytokine-driven pathway of activation allows iNKT cell recognition of pathogens that express TLR ligands but appear to lack CD1d-presented lipid antigens, such as viruses or the Gram-negative bacterium *Salmonella typhimurium* (Brigl et al., 2003; Mattner et al., 2005; Tyznik et al., 2008; Wesley et al., 2008).

The current model suggests that, dependent on the expression of antigens by the microbe, iNKT cell activation during microbial infection is cognate, foreign antigen driven, or innate cytokine driven (Mattner et al., 2005; Tupin et al., 2007;

Brigl and Brenner, 2010). In this paper, we investigated the relative contributions of microbial antigen– versus cytokine-driven pathways in iNKT cell activation using a large panel of diverse bacterial pathogens, several of which are known to express iNKT cell antigens and/or have been shown to require iNKT cells for protective immunity. Unexpectedly, we found that iNKT cell IFN-γ production was dominantly dependent on innate mechanisms with TLR-mediated signaling and the production of IL-12 by APCs, irrespective of whether or not bacteria express CD1d-presented iNKT cell antigens. Furthermore, high levels of IL-12 receptor were expressed by iNKT cells, readying them for rapid cytokine-mediated stimulation. Thus, our data suggest that innate signals, together with cytokine-driven activation, are the dominant pathway enabling rapid iNKT cell responses to diverse microbial infections.

RESULTS

Antigen- and cytokine-driven pathways of iNKT cell activation

Studies using only NKT cell hybridomas do not adequately model the NKT cell activation mechanism that may occur in vivo because such systems lack the potential to respond to both antigen and cytokine signals. To investigate the mechanisms of iNKT cell activation by microbes, we used a system with primary mouse iNKT cell lines and BM-derived DCs that is able to respond to a variety of stimuli (Chiba et al., 2009). iNKT cell lines incubated with DCs and stimulated with the CD1d-presented microbial GSL antigen GSL-1, which is found in *Sphingomonas* spp. (Kinjo et al., 2005; Mattner et al., 2005; Sriram et al., 2005), produced large amounts of IFN-γ (Fig. 1 A, left). This IFN-γ response was dependent on recognition of CD1d by iNKT cell lines, as use of CD1d-deficient DCs resulted in markedly reduced IFN-γ secretion (Fig. 1 A). To determine the requirement for TLR- and cytokine-mediated stimulation for the activation of iNKT cell lines, we performed experiments using DCs deficient in the adaptor protein MyD88 or the production of IL-12, as well as blocking antibodies against IL-12. Stimulation of iNKT cell lines with GSL-1 was not altered when DCs deficient in MyD88 were used (Fig. 1 B) and was only marginally reduced when IL-12–deficient DCs were used or when mAbs to IL-12 were added to the cultures (Fig. 1 C). Thus, iNKT cell activation by a microbial lipid antigen required CD1d expression and was essentially independent of TLR signaling or IL-12 production by APCs. The slightly reduced iNKT cell IFN-γ response to GSL-1 in the presence of IL-12–deficient DCs or after addition of mAb against IL-12 was likely the result of a lack of IL-12–mediated amplification of iNKT cell IFN-γ production that has been noted after CD40L-mediated stimulation of DCs by antigen-activated iNKT cells (Tomura et al., 1999).

In contrast to their direct stimulation by CD1d-presented microbial lipid antigens, iNKT cells can also be stimulated with TLR agonists in the presence of DCs by a self-antigen– and cytokine-driven pathway that does not require the cognate recognition of microbial antigens by iNKT cells (Brigl et al., 2003; Nagarajan and Kronenberg, 2007; Paget et al., 2007; Salio et al., 2007). Indeed, iNKT cell lines incubated with WT DCs and stimulated with the TLR agonists LPS (TLR4) or CpG (TLR9) produced copious amounts of IFN-γ (Fig. 1 A, second and third panel). To determine the requirements for a

Table I. Bacteria used in this study

Organism	Strain	Antigens	Role of iNKT in infection	Reference
Gram-positive				
S. pneumoniae	URF918	GlcDAG, GalGlcDAG	Protective	Burrows et al., 2009; Kawakami et al., 2003
L. monocytogenes	10403S	N.d.	Protective	Arrunategui-Correa and Kim, 2004
S. aureus	25923[a]	N.d.	N.d.	Brigl et al., 2003
Gram-negative				
P. aeruginosa	D4	N.d.	Protective	Nieuwenhuis et al., 2002
S. typhimurium	MT110	N.d.	Not protective	Berntman et al., 2005; Brigl et al., 2003
E. coli	25922[a]	N.d.	N.d.	
α-Proteobacteria				
S. capsulata	14666[a]	GSL-1 (GlcAGSL)	Protective	Mattner et al., 2005
N. aromaticivorans	700278[a]	GSL-1	Induction of PBC	Mattner et al., 2008
S. yanoikuyae	51230[a]	GSL-1	Protective	Kinjo et al., 2005
Spirochetes				
B. burgdorferi	N40	BbGL-II (GalDAG)	Protective	Kinjo et al., 2006; Kumar et al., 2000; Tupin et al., 2008
Mycobacteria				
M. tuberculosis	H37Rv	PIM_4[b]	Not protective	Behar et al., 1999; Fischer et al., 2004

Description of bacteria used in this study, microbial lipid antigens, if any, described for the respective bacterium, and role of iNKT cells during infection.
GlcAGSL, glucuronic acid containing GSL; BbGL-II, *B. burgdorferi* glycolipid II; GalDAG, galactosyl diacylglycerol; PIM_4, phosphatidylinositol mannoside 4; N.d., not described; PBC, primary biliary cirrhosis.
[a]American Type Culture Collection number.
[b]Antigenic activity of this lipid could not be confirmed by some investigators (Kinjo et al., 2006).

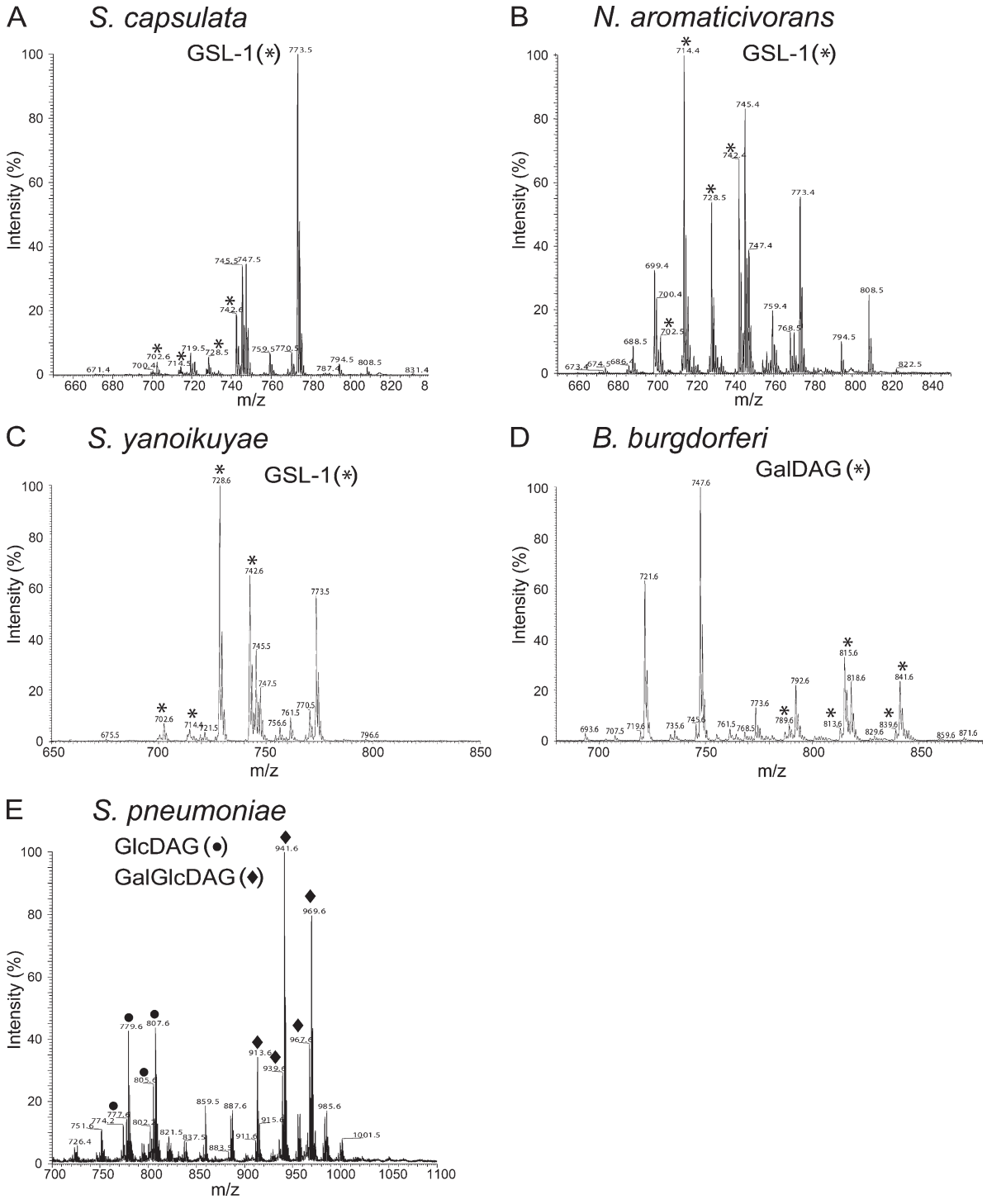

Figure 2. Detection of microbial lipid antigens expressed by bacteria. Lipids were extracted from bacteria and analyzed by ESI mass spectrometry. (A–C) $[M - H]^-$ adducts of the GSL-1 (GlcAGSL) antigen in *S. capsulata*, *N. aromaticivorans*, and *S. yanoikuyae*. (D) $[M + CH3COO]^-$ adducts of the GalDAG (BbGL-II) antigen in *B. burgdorferi*. (E) $[M + Na]^+$ adducts of the GlcDAG and GalGlcDAG antigens in *S. pneumoniae*. For annotation of sphingosine and acyl chain composition see Table S1. For corresponding LC and MS data of lipid antigens expressed by bacteria see SI Fig. 1.

CD1d–TCR interaction, TLR signaling, and IL-12 stimulation after activation of iNKT cells with TLR agonists, we used DCs from CD1d-, MyD88-, or IL-12–deficient mice and blocking antibodies against IL-12. Using CD1d-deficient DCs, TLR agonist–induced IFN-γ production by iNKT cell lines was significantly reduced in response to LPS or CpG, respectively, compared with stimulation in the presence of WT DCs (Fig. 1 A). Because no exogenous microbial antigens

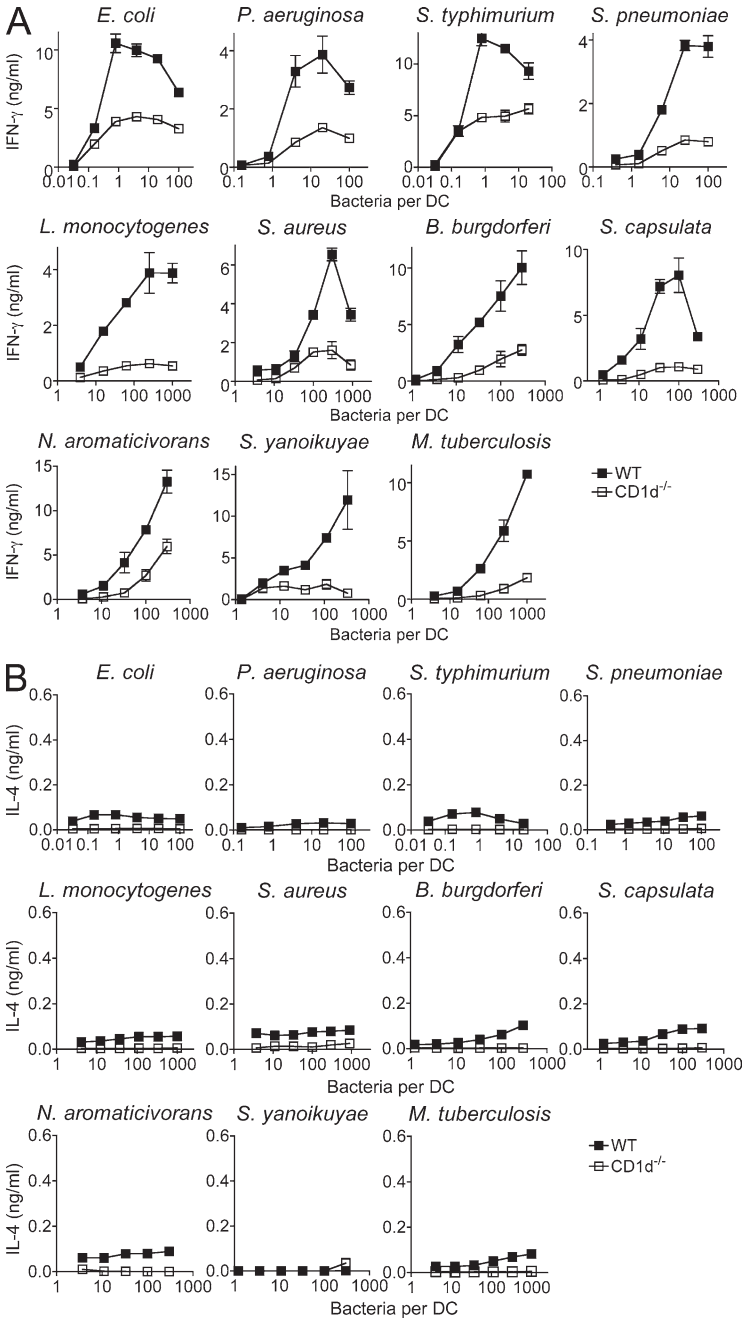

Figure 3. Cytokine responses of iNKT cells to diverse bacteria. iNKT cell lines were cultured with WT (filled squares) or CD1d-deficient (open squares) DCs in the presence of heat-inactivated bacteria. (A and B) IFN-γ (A) and IL-4 (B) concentrations were measured in culture supernatants by ELISA after 16–24 h. Data represent means of duplicate values ± SD and are representative of at least three independent experiments.

were present under these conditions, recognition of CD1d-presented cellular self-antigens appeared to be required for iNKT cell activation, as has been previously noted (Brigl et al., 2003). When MyD88-deficient DCs were used during stimulation of iNKT cell lines, IFN-γ production in response to LPS or CpG was markedly reduced (Fig. 1 B). Furthermore, iNKT cell activation in response to LPS or CpG was strikingly reduced when DCs deficient in IL-12 production were used and when mAb to IL-12 was added to the cultures (Fig. 1 C). In response to antigens, iNKT cells are also known to secrete IL-4. After stimulation with GSL-1, iNKT cell lines produced IL-4 in a CD1d-dependent and MyD88- and IL-12–independent manner (Fig. 1, D and E). In response to LPS or CpG, no significant amounts of IL-4 were detected (Fig. 1, D and E). Thus, TLR agonist–induced IFN-γ secretion by iNKT cell lines in the presence of DCs was driven by recognition of CD1d-presented self-antigens and IL-12, which are produced by APCs after TLR-mediated activation, whereas antigen-driven activation results in TLR- and IL-12–independent IFN-γ and IL-4 secretion.

Expression of microbial iNKT cell antigens

To examine the mechanism of iNKT cell activation in response to bacterial infection, we assembled a panel of 11 bacteria, 5 of which are known to express CD1d-presented iNKT cell antigens (Table I). The diverse panel of bacteria was selected to include clinically important pathogens such as the Gram-positive bacteria *S. pneumoniae*, *Listeria monocytogenes* and *Staphylococcus aureus*, the Gram-negative bacteria *Pseudomonas aeruginosa*, *S. typhimurium*, and *E. coli*, the spirochete *B. burgdorferi*, the mycobacterium *Mycobacterium tuberculosis*, and the α-proteobacteria *Sphingomonas capsulata*, *Novosphingobium aromaticivorans*, and *Sphingomonas yanoikuyae*. Members of the class of α-proteobacteria are known to cause opportunistic infections in humans and have been associated with the induction of autoimmunity (Mohammed and Mattner, 2009; Ryan and Adley, 2010). Furthermore, iNKT cells are critical in mice for protective immunity to infection with *S. pneumoniae*, *B. burgdorferi*, *P. aeruginosa*, *S. capsulata*, and *L. monocytogenes*, and iNKT cells have been implicated in the development of primary biliary cirrhosis in a mouse model after infection with *N. aromaticivorans* (see Table I for summary and references).

From among the panel of bacteria, microbial CD1d-presented lipid antigens that are capable of stimulating iNKT cells have been described for *S. capsulata*, *N. aromaticivorans*, and *S. yanoikuyae* (all three GSL-1), *B. burgdorferi* (BbGL-II), and *S. pneumoniae* (monoglycosyl- and diglycosyldiacylglycerol; for details and references see Table I). To confirm that bacteria used in this study indeed expressed iNKT cell antigens, we isolated polar lipids from *S. capsulata*, *N. aromaticivorans*, *S. yanoikuyae*, *B. burgdorferi*, and *S. pneumoniae* and subjected the lipids to electrospray mass spectrometry analysis. Lipids from *S. capsulata*, *N. aromaticivorans*, and *S. yanoikuyae* yielded ions representing the $[M - H]^-$ ions of the antigenic α-glucuronosylceramide GSL-1 (Fig. 2, A–C; and Table S1).

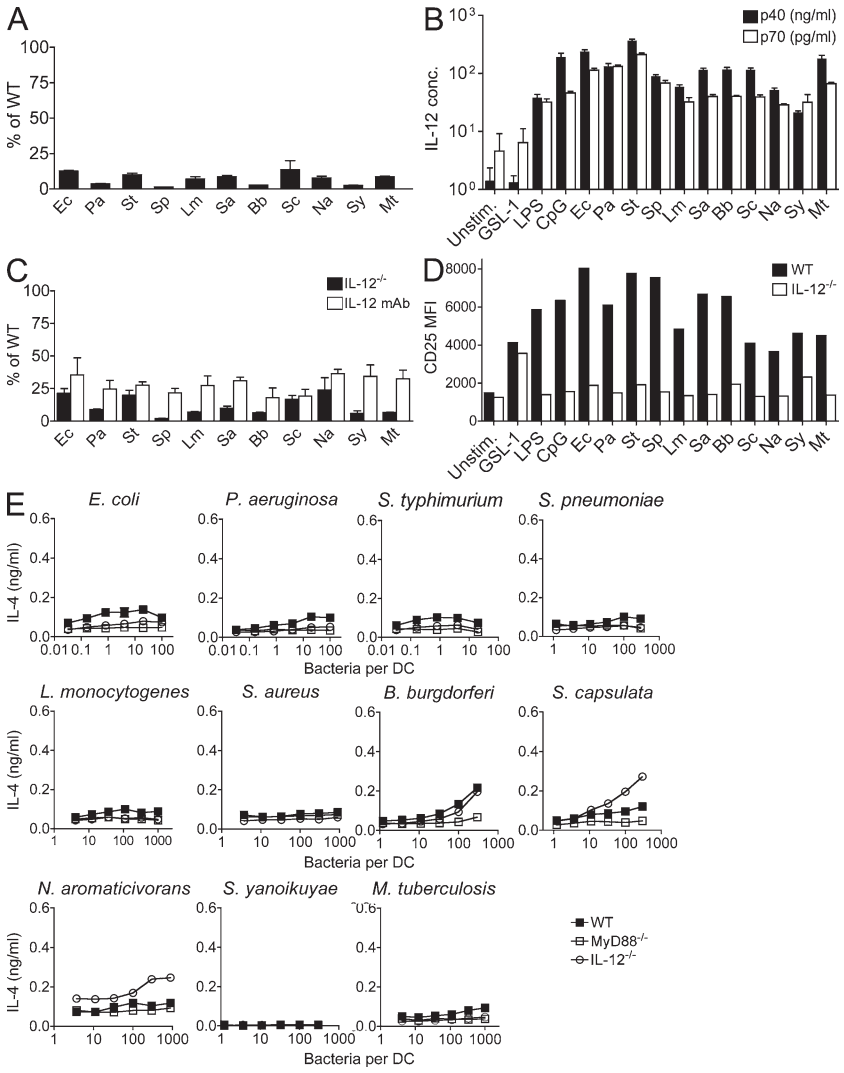

Figure 4. Mechanism of iNKT cell activation in response to diverse bacteria (A) iNKT cell lines were cultured with WT or MyD88-deficient DCs in the presence of heat-inactivated bacteria. Data for IFN-γ secretion in culture supernatants are shown as percent inhibition comparing MyD88-deficient to WT DCs after stimulation with 10 bacteria per DC for *E. coli* (Ec), *P. aeruginosa* (Pa), and *S. typhimurium* (St) and with 100 bacteria per DC for *S. pneumoniae* (Sp), *L. monocytogenes* (Lm), *S. aureus* (Sa), *B. burgdorferi* (Bb), *S. capsulata* (Sc), *N. aromoticivorans* (Na), *S.yanoikuyae* (Sy), and *M. tuberculosis* (Mt). Data are summarized from four independent experiments (mean ± SD). (B) Concentration of IL-12p40 (ng/ml, filled bars) and IL-12p70 (pg/ml, open bars) measured by ELISA in culture supernatants of DCs stimulated with microbial products or heat-killed bacteria as in Fig. 1 and Fig. 4 A, respectively (mean ± SD). No IL-18 was detected in culture supernatants and IFN-β concentrations were not different between unstimulated and stimulated conditions (not depicted). (C) iNKT cell lines were cultured with WT or IL-12p35–deficient DCs in the presence of stimuli as described in A. Data are shown as percentage of IFN-γ secretion in culture supernatants comparing IL-12–deficient to WT DCs after stimulation (filled bars; mean ± SD). Open bars represent the percentage of IFN-γ secretion in the presence of 10 µg/ml blocking mAb against IL-12. No significant inhibition was observed with isotype-matched control antibodies (not depicted). Data are summarized from two independent experiments. (D) Expression of the early activation marker CD25 by iNKT cells after 24 h of co-culture with WT or IL-12–deficient DCs in the presence of stimuli as in A. GSL-1 was used at 2 µg/ml, LPS at 10 ng/ml, and CpG at 2 µg/ml. (E) IL-4 production by iNKT cells in response to heat-inactivated bacteria using WT (filled squares), MyD88-deficient (open squares), or IL-12–deficient (open circles) DCs. Experiments were performed as in Fig. 1 (D and E). IL-4 concentrations in culture supernatants were measured by ELISA. Data are presented as means of duplicate values ± SD and are representative of at least three independent experiments.

For *B. burgdorferi* lipids, ions corresponding to the $[M + CH3COO]^-$ adduct ions of α-galactosyldiacylglycerol, the BbGL-II antigen, were detected (Fig. 2 D and Table S1). In positive-ion mode, the lipids from *S. pneumoniae* gave rise to $[M + Na]^+$ ions corresponding to the α-glucosyldiacylglycerol (GlcDAG), together with ions corresponding to the α–galactosyl GlcDAG (GalGlcDAG; Fig. 2 E and Table S1). The structural assignments for individual lipid species were supported by multiple-stage ion-trap tandem mass spectrometry (Table S1 and not depicted). Further analysis of the bacterial lipids by liquid chromatography, light scattering detection, and mass spectrometry analysis confirmed the presence of the respective antigenic lipid species in these five bacteria and suggested that all bacteria expressed significant amounts of the respective antigens (Fig. S1). Thus, 5 of the 11 bacteria used in this study expressed known iNKT cell lipid antigen species as expected. No lipids capable of stimulating iNKT cells directly have so far been described for the other 6 bacteria.

Mechanism of bacteria-induced iNKT cell activation

We next examined the mechanism of iNKT cell activation in response to the 11 bacteria described in the previous section. iNKT cell lines incubated with WT DCs and stimulated with any of the 11 heat-killed bacteria produced substantial amounts of IFN-γ (Fig. 3 A). iNKT cell activation induced by heat-killed bacteria required a CD1d-TCR interaction, as IFN-γ production by iNKT cell lines in the presence of CD1d-deficient DCs and any of the 11 heat-killed bacteria was reduced by a mean of 50–80%, compared with stimulation in the presence of WT DCs (Fig. 3 A). Little or no IL-4 was generated by iNKT cells after stimulation with most of the heat-killed bacteria, whereas stimulation with *B. burgdorferi*, *S. capsulata*, *N. aromaticivorans*, and *S. yanoikuyae* resulted in production of low amounts of IL-4 (Fig. 3 B and not depicted). The amounts of IL-4 detectable in culture supernatants varied substantially between experiments and were generally reduced after repeated stimulation of iNKT cell lines, and no

significant amounts of IL-4 were detected with iNKT cells isolated directly from Vα14 TCR transgenic mice (unpublished data).

Next, we determined if iNKT cell activation by heat-killed bacteria required TLR-mediated signaling by the APCs. iNKT cell lines incubated with MyD88-deficient DCs plus any of the 11 heat-killed bacteria resulted in reduced IFN-γ secretion when compared with WT DCs (Fig. 4 A and Fig. S2). Thus, whether the bacteria used for stimulation expressed known microbial lipid antigens, iNKT cell IFN-γ secretion was critically dependent on TLR-mediated signaling by DCs. This result suggested that iNKT cell activation in response to bacteria expressing microbial iNKT cell antigens might not be mediated by TCR recognition of the microbial lipid antigens and instead might be cytokine-driven.

IL-12, IL-18, and type I IFNs have been implicated in activating iNKT cells after TLR-mediated stimulation of DCs in the absence of iNKT cell recognition of CD1d-presented microbial lipid antigens (Brigl et al., 2003; Mattner et al., 2005; Nagarajan and Kronenberg, 2007; Paget et al., 2007; Salio et al., 2007). We determined if any of these cytokines were generated by DCs after stimulation with the panel of heat-killed bacteria by measuring the concentration of IL-12, IL-18, and IFN-β in supernatants from DCs cultured with TLR agonist, GSL-1, or any of the 11 heat-killed bacteria. Significant quantities of IL-12p40 and IL-12p70, but not of IL-18 or IFN-β, were detected in supernatants of DCs exposed to LPS, CpG, or any of the 11 heat-killed bacteria (Fig. 4 B and not depicted). In contrast, no IL-12p40 or IL-12p70 was detected in supernatants of DCs cultured in the presence of the GSL-1 antigen. Then, we determined whether IL-12 was required for IFN-γ secretion by iNKT cell lines in response to stimulation with heat-killed bacteria. Indeed, incubation of iNKT cell lines with IL-12–deficient DCs in the presence of any of the 11 heat-killed bacteria resulted in substantially reduced IFN-γ production compared with stimulation in the presence of WT DCs (Fig. 4 C and Fig. S2). Similarly, addition of blocking mAb against IL-12 reduced iNKT cell IFN-γ secretion after stimulation with any of the 11 heat-killed bacteria (Fig. 4 C). In contrast, stimulation of iNKT cell lines with IL-18–deficient DCs or after stimulation of iNKT cell/DC co-cultures in the presence of antibodies blocking type I IFN signaling did not show a requirement for IL-18 or type I IFNs, respectively, in response to any of the 11 heat-killed bacteria (Fig. S3, A and B). As described, MyD88- and IL-12–deficient DCs were capable of presenting the purified GSL-1 antigen to stimulate iNKT cell lines but were not capable of eliciting IFN-γ secretion by iNKT cell lines in response to the TLR agonists LPS and CpG (Fig. 1). We next determined if expression of the early activation marker CD25 on iNKT cells after stimulation was dependent on IL-12. Expression levels of CD25 were increased after stimulation with GSL-1, TLR agonists, or heat-killed bacteria (Fig. 4 D and Fig. S4). Stimulation with TLR agonists or heat-killed bacteria, but not with GSL-1, resulted in decreased expression on CD25 when DCs deficient in IL-12 were used. Similar results were obtained for the early activation marker CD69 (unpublished data). iNKT cell IFN-γ production and CD25 expression were also both reduced on day 2 after stimulation with antigen, TLR agonists, or heat-killed bacteria, suggesting that iNKT cell activation in the absence of IL-12 was not delayed (Fig. S5, A and B). The weak IL-4 responses observed after

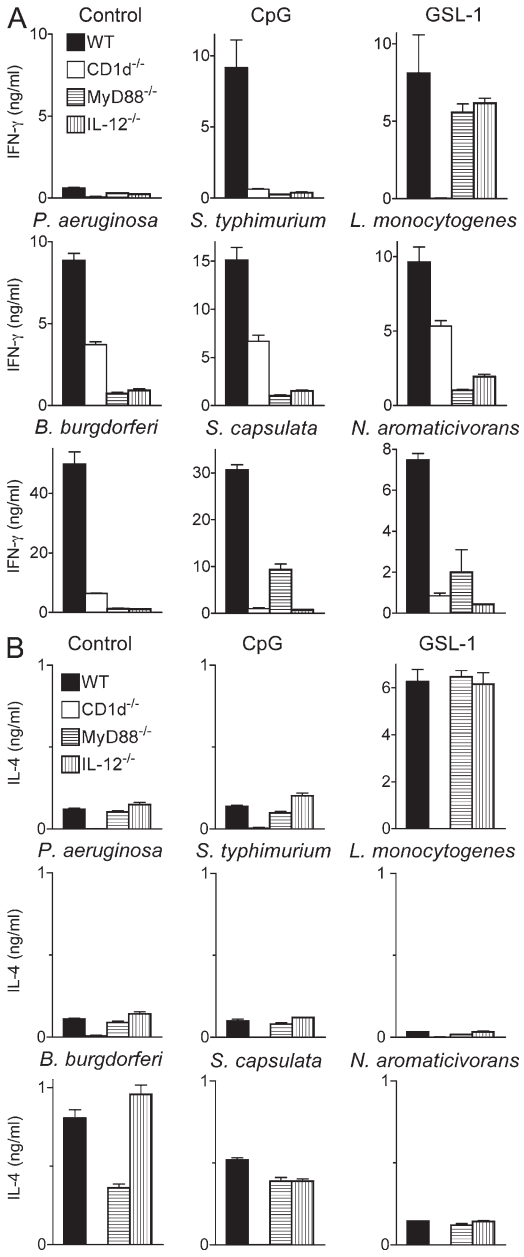

Figure 5. iNKT cell response to live in vitro infection. DCs were incubated with 2 μg/ml CpG, 10 μg/ml GSL-1, *P. aeruginosas*, *S. typhimurium* (both 10 live bacteria per DC), *L. monocytogenes*, *B. burgdorferi*, *S. capsulata*, or *N. aromaticivorans* (all 100 live bacteria per DC) for 3 h, followed by washing and plating with iNKT cell lines. (A and B) IFN-γ (A) or IL-4 (B) concentrations were measured in culture supernatants by ELISA after 16–24 h. Data are displayed as means of triplicate measurements ± SD and are representative of at least two independent experiments.

stimulation with *Borrelia* and *Sphingomonas* spp. showed a trend toward being reduced when MyD88-deficient DCs were used and toward being increased when IL-12–deficient DCs were used (Fig. 4 E). iNKT cells freshly isolated from WT mice by tetramer sorting also showed a strong dependence of the iNKT cell IFN-γ production on MyD88 and IL-12, whereas little or no IL-4 was generated (Fig. S6, A and B). The CD1d-dependence of TLR agonist- and bacteria-induced activation of freshly isolated iNKT cell was reduced compared with what was observed using iNKT cell lines, which is likely the result of stimulation of iNKT cells using TCR-β antibodies and tetramer for their purification by cell sorting, as has been observed previously (Matsuda et al., 2003; Nagarajan and Kronenberg, 2007).

To determine if alterations in CD1d expression levels by the APCs may contribute to the observed iNKT cell activation, CD1d expression levels were determined on WT DCs after exposure to heat-killed bacteria. Stimulation with the TLR agonists LPS and CpG and all 11 of the heat-killed bacteria, but not GSL-1, resulted in increased expression of CD1d by DCs (Fig. S7, A and B). The increased expression of CD1d after stimulation was absent in the presence of MyD88-deficient DCs, suggesting that TLR-mediated signaling was required. Thus, iNKT cell activation by a wide variety of heat-killed bacterial pathogens in vitro, including bacteria which expressed CD1d-presented microbial lipid antigens, such as *Sphingomonas* spp., *B. burgdorferi*, *M. tuberculosis*, or *S. pneumoniae*, was dominantly dependent on TLR signaling and IL-12–driven stimulation.

iNKT cell activation after live in vitro infection

To further investigate the mechanism of iNKT cell activation in response to DCs infected with live bacteria, we used an in vitro live infection model with three microorganisms shown to express iNKT cell antigens (*B. burgdorferi*, *S. capsulata*, and *N. aromaticivorans*; Table I) and three microorganisms for which iNKT cell antigens have not been described (*P. aeruginosa*, *S. typhimurium*, and *L. monocytogenes*). DCs were infected with bacteria for 3 h and infected DCs were then co-cultured with iNKT cell lines, resulting in production of substantial amounts of IFN-γ for all six bacteria tested (Fig. 5 A). To determine the relative requirements for a CD1d–TCR interaction, TLR signaling, and IL-12 stimulation after activation of iNKT cells with infected DCs, we used DCs from CD1d-, MyD88-, or IL-12–deficient mice. Infection of DCs deficient in CD1d, MyD88, or IL-12 production with *P. aeruginosa*, *S. typhimurium*, *L. monocytogenes*, *B. burgdorferi*, *S. capsulata*, or *N. aromaticivorans* resulted in reduced IFN-γ production by iNKT cell lines, compared with infection of WT DCs (Fig. 5 A). Live in vitro infection with *B. burgdorferi*, and to a lesser extent *S. capsulata*, resulted in the production of IL-4 (Fig. 5 B). This IL-4 production was largely MyD88 dependent but IL-12 independent. Thus, similar to the results obtained after stimulation with heat-killed bacteria, iNKT cell IFN-γ production in response to DCs infected with live bacteria was dependent on CD1d recognition, TLR-signaling, and IL-12 stimulation, irrespective of the expression of iNKT cell antigens by the bacteria. This suggested that iNKT cell activation in response to infected DCs was dominantly cytokine driven and that TLR-mediated signals were required, whereas CD1d-mediated signals played a variable role.

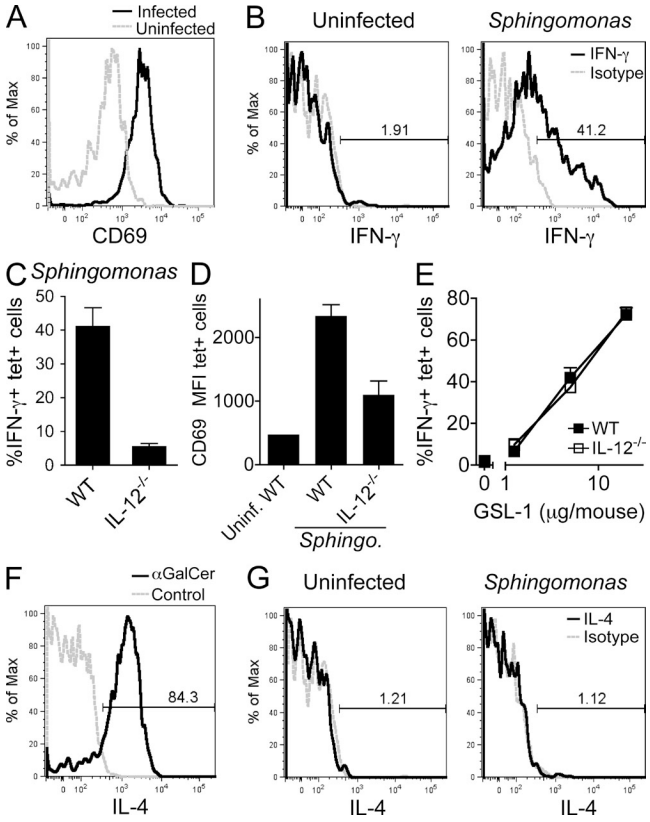

Figure 6. In vivo activation of iNKT cells during *S. yanoikuyae* infection. (A) CD69 surface staining on CD1d tetramer–positive iNKT cells isolated from the livers of uninfected WT mice (dotted line) or 18 h after i.v. infection with *S. yanoikuyae* (bold line). (B) IFN-γ secretion by CD1d tetramer–positive liver iNKT cells from uninfected animals (left, bold line) or 18 h after i.v. infection with *S. yanoikuyae* (right, bold line) in comparison with staining with isotype control antibodies (dotted line). (C and D) Comparison of IFN-γ secretion (C) and CD69 expression (D) by CD1d tetramer–positive liver iNKT cells in WT and IL-12–deficient mice 18 h after i.v. infection with *S. yanoikuyae* (mean ± SD). (E) WT or IL-12-deficient mice were injected i.v. with 20, 5, or 1.25 μg GSL-1, and IFN-γ secretion by CD1d tetramer–positive liver iNKT cells was determined after 80 min. Means ± SD are shown. Data are pooled from three independent experiments and represent four to six mice per condition. (F) IL-4 secretion by liver iNKT cells 45 min after injection of 2 μg α-GalCer. Similar results were observed with GSL-1 (not depicted). (G) IL-4 secretion by liver iNKT cells from uninfected animals or 18 h after i.v. infection with *S. yanoikuyae* in comparison with staining with isotype control antibodies. Data are representative of two independent experiments using three to four mice per condition.

In vivo activation of iNKT cells during *Sphingomonas* infection

Next, we analyzed iNKT cell activation in a mouse model of *Sphingomonas* infection. Mononuclear cells were isolated from

WT mice 18 h after intravenous infection with *S. yanoikuyae* and iNKT cells were identified using CD1d tetramers. CD1d tetramer–positive iNKT cells from livers of uninfected mice stained at intermediate intensity for the early activation marker CD69 (mean fluorescence intensity [MFI], 465 ± 13) and, 18 h after infection, expression of CD69 increased to an MFI of 2,990 ± 639 (Fig. 6 A). IFN-γ secretion by iNKT cells showed that a mean of 41 ± 14% of the CD1d tetramer–positive iNKT cells were positive 18 h after infection, compared with a mean of 2 ± 0.1% in uninfected controls (Fig. 6 B). Thus, iNKT cells became rapidly activated after *S. yanoikuyae* infection. To assess whether IL-12 was required for the in vivo activation of iNKT cells during *S. yanoikuyae* infection, as suggested by our in vitro experiments, we compared IFN-γ secretion and CD69 expression by liver iNKT cells in WT versus IL-12–deficient mice after infection. 18 h after infection, IL-12 deficiency resulted in reduced IFN-γ secretion and CD69 expression (Fig. 6, C and D).

To compare the requirement for IL-12 in iNKT cell IFN-γ secretion after infection with *S. yanoikuyae* bacteria that express the GSL-1 antigen with iNKT cell activation induced by the GSL-1 antigen, we analyzed iNKT cell activation after stimulation with purified GSL-1 antigen. After injection of a range of doses of GSL-1 antigen, IFN-γ secretion by CD1d tetramer–positive liver iNKT cells was similar in WT or IL-12–deficient mice (Fig. 6 E). IL-4 production by iNKT cell in vivo was readily observed after injection of the α-galactosylceramide (GalCer) antigen; however, during infection with *S. yanoikuyae*, *S. capsulata*, or *N. aromaticivorans* no significant amounts of IL-4 were detected in either WT or IL-12–deficient mice (Fig. 6, F and G; and not depicted). Thus, iNKT cell activation after infection with a GSL-1–expressing microbe is dependent on IL-12, whereas iNKT cell activation with a microbial antigen alone does not require IL-12.

iNKT cell activation during *S. pneumoniae* infection

Next, we examined the role of iNKT cells after infection with *S. pneumoniae*. Infection of WT and iNKT cell–deficient Jα18$^{-/-}$ mice with *S. pneumoniae* showed reduced survival of iNKT cell–deficient Jα18$^{-/-}$ mice (Fig. 7 A). Between days 6 and 12 after infection, >50% of Jα18$^{-/-}$ mice succumbed to infection, whereas none of the WT animals died. Similar results were obtained in CD1d-deficient animals (Fig. S8 A). Bacterial burden in lungs of infected WT or Jα18$^{-/-}$ animals were comparable on day 3 after infection. However, on day 6 after infection, bacterial burden in lungs of Jα18$^{-/-}$ were ∼1,000-fold higher when compared with WT animals (Fig. 7 B). Thus, iNKT cells were critically important for protective immunity after pulmonary infection with *S. pneumoniae*.

To examine iNKT cell activation after pulmonary infection with *S. pneumoniae*, lymphocytes were isolated from lungs of infected and uninfected animals and iNKT cells were analyzed by flow cytometry for surface expression of the activation marker CD69 and for secretion of IFN-γ. Expression of CD69 was intermediate on iNKT cells from uninfected mice and increased on days 2, 4, and 7 after infection (Fig. 7 C).

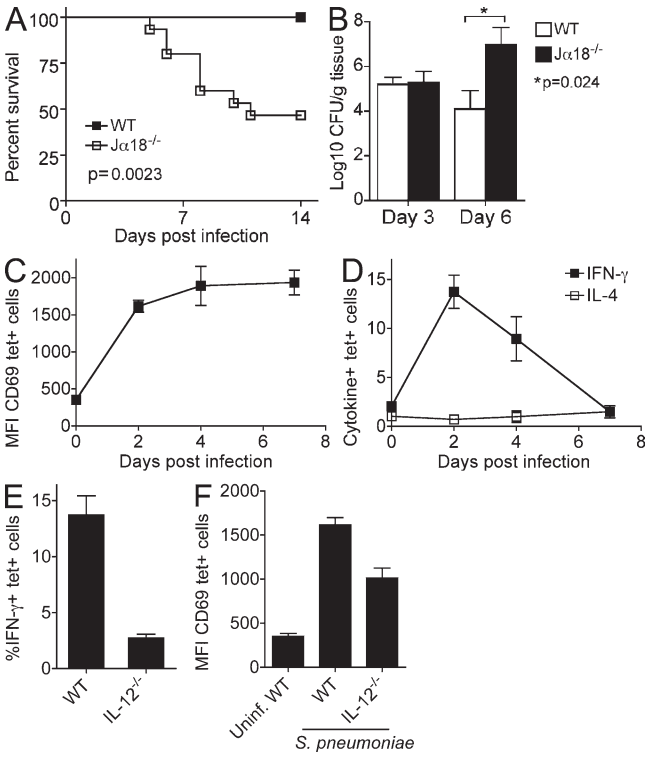

Figure 7. In vivo activation of iNKT cells during *S. pneumoniae* infection. WT or Jα18-deficient mice were infected intranasally with *S. pneumoniae*. (A) Survival was recorded daily for 2 wk for WT (filled squares; $n = 9$) and Jα18$^{-/-}$ (open squares; $n = 9$) mice. Results are representative of two independent experiments. (B) The number of CFU was determined in lung tissues of WT (open bars; $n = 6$) or Jα18$^{-/-}$ (filled bars; $n = 6$) on days 3 and day 6 after infection. Data are pooled from two independent experiments (mean ± SD). (C and D) Lymphocytes were isolated from lungs of mice infected intratracheally with *S. pneumoniae* or from uninfected mice and analyzed by flow cytometry. Staining for surface expression of CD69 (C) or secreted IFN-γ or IL-4 (D) on CD1d tetramer–positive lymphocytes are shown. Data represent means ± SD ($n = 6$) and are pooled from two independent experiments. (E and F) WT or IL-12p35–deficient mice were infected intratracheally with *S. pneumoniae* and secretion of IFN-γ (E) or expression of CD69 by iNKT cells (F) was determined as described in C and D on day 2 after infection. Data represent means ± SD for three to four mice per group and one experiment of two similar experiments is shown.

Thus, iNKT cells became activated rapidly after *S. pneumoniae* infection. Cytokine secretion assays showed significant IFN-γ secretion by iNKT cells on days 2 and 4 after infection that returned to baseline by day 7 after infection (Fig. 7 D). No significant production of IL-4 by iNKT cells was observed in either WT or IL-12–deficient mice during infection at any of the time points examined (Fig. 7 D and not depicted). Thus, iNKT cells became activated rapidly and expressed IFN-γ early during pulmonary infection with *S. pneumoniae*.

Next, we determined whether IL-12 was required for the activation of iNKT cells during *S. pneumoniae* infection, as was the case in the in vitro experiments described in the previous paragraphs. IL-12 deficiency resulted in reduced IFN-γ secretion and CD69 expression on days 2 and 4 after infection

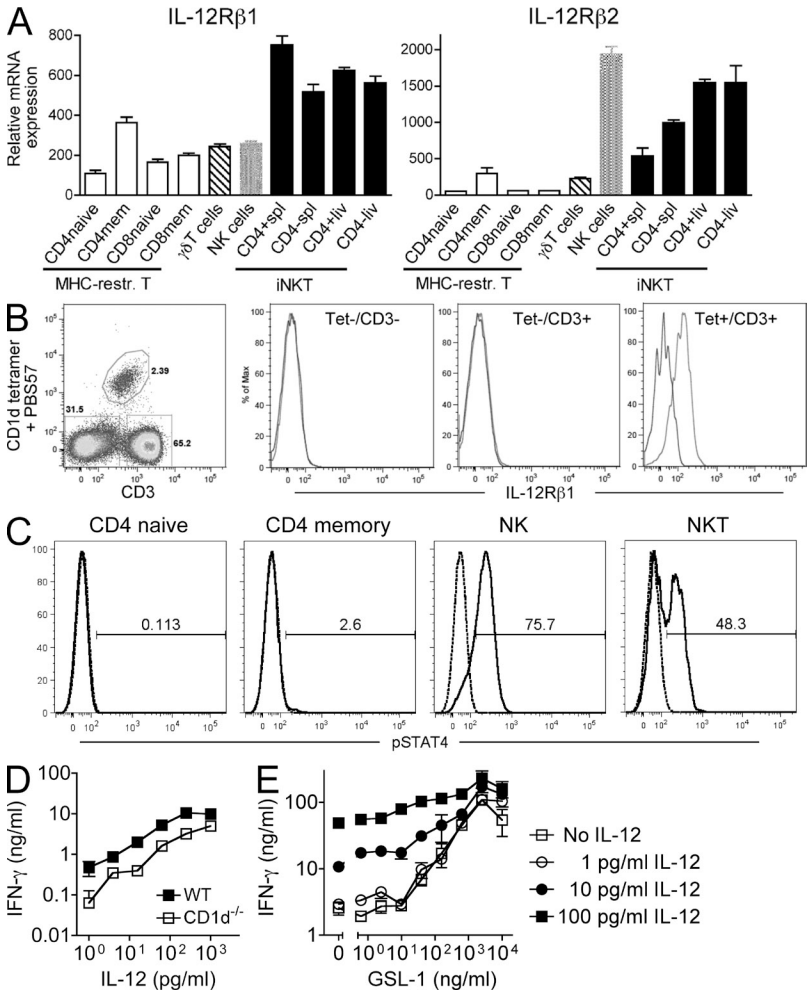

Figure 8. Expression and function of IL-12 receptor on iNKT cells. (A) IL-12Rβ1 (left) and IL-12Rβ2 (right) mRNA expression in purified splenocyte subsets or iNKT cell subsets purified from spleen or liver was assessed by microarray. Data are representative of two to four independent experiments and are shown as mean ± SD. (B) Flow cytometry staining of $CD19^-$ splenocytes with CD1d tetramers and anti-CD3 antibodies (left). The $tetramer^-/CD3^-$ gate contains NK cells, the $tetramer^-/CD3^+$ gate contains MHC-restricted CD4 and CD8 αβT cells and γδT cells, and the $tetramer^+/CD3^+$ gate contains iNKT cells. Surface expression of IL-12Rβ1 was determined by flow cytometry on gated lymphocyte subpopulations. (C) Naive and memory CD4 T cells, NK cells, and iNKT cells were isolated from spleens of WT mice and purified by cell sorting. Cells were stimulated in the presence of 1 ng/ml recombinant IL-12 for 1 h and subsequently stained with antibodies against the phosphorylated form of STAT4 (solid lines) or isotype control antibodies (dotted lines). (D) iNKT cell lines were incubated with WT (filled squares) or $CD1d^{-/-}$ (open squares) DCs in the presence of various concentrations of recombinant IL-12. Data are presented as means of duplicate values ± SD and are representative of at least three independent experiments. (E) iNKT cell lines were incubated with WT DCs and various concentrations of the microbial lipid antigen GSL-1 in the absence or presence of various concentrations of recombinant IL-12. IFN-γ concentrations were measured in culture supernatants by ELISA after 16–24 h. Data are presented as means of triplicate values ± SD and are representative of two independent experiments.

(Fig. 7, E and F; and Fig. S8, B and C). Thus, most of the iNKT cell activation and IFN-γ production during *S. pneumoniae* infection in vivo was critically dependent on IL-12.

Expression and function of IL-12 receptor on iNKT cells

Responsiveness to IL-12 correlates with expression of IL-12 receptor (IL-12R) on NK and activated T cells (Presky et al., 1996; Trinchieri, 2003). To determine the expression of IL-12R by iNKT cells, we took complementary approaches. First, expression of mRNA for the two IL-12R subunits, IL-12Rβ1 and IL-12Rβ2, was determined by microarray analysis in iNKT cells and other lymphocyte subsets. IL-12Rβ1, which is responsible for binding IL-12, was expressed at very low levels by naive CD4 and CD8 T cells, memory CD8 T cells, γδT cells, and NK cells, whereas slightly higher levels were detected in memory CD4 T cells (Fig. 8 A). In contrast, much higher levels of IL-12Rβ1 mRNA were detected in both $CD4^+$ and $CD4^-$ iNKT cells isolated from spleen or liver. IL-12Rβ2, which is required for IL-12R–mediated signaling, was expressed at very low levels by naive CD4 and CD8 T cells, memory CD8 T cells, and γδT cells, whereas slightly higher levels were expressed by memory CD4 T cells (Fig. 8 A). In comparison, much higher levels of IL-12Rβ2 mRNA were detected in NK cells. Intermediate levels of IL-12Rβ2 were expressed by $CD4^+$ and $CD4^-$ iNKT cells isolated from spleen, and high levels, similar to those detected on NK cells, were detected in $CD4^+$ and $CD4^-$ liver iNKT cells. Second, we determined if the expression levels of IL-12R mRNA correlated with protein expression. Using CD1d tetramers to detect iNKT cells, we found that expression of IL-12Rβ1 could be detected exclusively on $tetramer^+/CD3^+$ lymphocytes (iNKT cells) but not on $tetramer^-/CD3^-$ lymphocytes (mainly NK cells) or $tetramer^-/CD3^+$ (containing MHC-restricted CD4 and CD8 αβT cells and γδT cells; Fig. 8 B).

The specific cellular effects of IL-12 are mainly a result of its ability to induce STAT4 activation. To determine if the constitutive expression of high levels of IL-12 receptor enabled prompt STAT4 activation in iNKT cells after IL-12 stimulation, we purified naive and memory CD4 T cells, NK cells, and iNKT cells by cell sorting and stimulated the purified cell populations with IL-12 for 1 h. Intracellular phosphorylation of STAT4 was assessed by antibody staining and flow cytometry. No STAT4 phosphorylation was detected in naive and memory CD4 T cells (Fig. 8 C). In contrast, phosphorylation of STAT4 was detected in a large number of NK cells and iNKT cells. Thus, unique among T cells but similar

to NK cells, high constitutive expression of functional IL-12 receptor appears to provide a mechanistic explanation for the ability of iNKT cells to rapidly secrete IFN-γ in response to IL-12–mediated stimulation.

Last, we sought to address how IL-12–mediated co-stimulation alters iNKT cell activation by CD1d-presented self- and microbial lipids. The addition of recombinant IL-12 to iNKT cell lines cultured in the presence of WT DCs resulted in notably increased IFN-γ secretion, as compared with the addition of IL-12 to iNKT cell lines co-cultured in the presence of CD1d-deficient DCs (Fig. 8 D). As no exogenous antigens were added in this system, the stimulation provided by CD1d recognition was likely a result of recognition of CD1d-presented self-lipids. Next, we determined how co-stimulation with IL-12 altered iNKT cell responses to microbial antigens. A cross-titration of GSL-1 and IL-12 revealed that in particular low concentrations of GLS-1 and low concentrations of IL-12 resulted in a synergistic stimulation of IFN-γ by iNKT cells (Fig. 8 E). Thus, weak TCR-mediated stimulation of iNKT cells with CD1d-present self- or microbial antigens was significantly amplified by IL-12, resulting in potent secretion of IFN-γ by iNKT cells.

DISCUSSION

iNKT cells have been shown to play important protective roles during several bacterial, parasitic, and viral infections. However, the central question of how they are activated by microbes is not fully explained. Because viruses lack lipid antigens and antigens have only been described for a few pathogens so far, it is important to determine how many diverse pathogens can activate iNKT cells. To investigate the mechanism of iNKT cell activation in response to infection, we used a large panel of diverse bacteria, including several that express known CD1d-presented iNKT cell lipid antigens. Unexpectedly, we found that, irrespective of the expression of CD1d-presented lipid antigens by the microbes, microbe-induced iNKT cell activation and IFN-γ production in vitro and in vivo was critically dependent on IL-12 released by DCs after TLR-mediated activation. Thus, our data do not support a recently proposed model in which iNKT cell activation during infection is driven by TCR-mediated recognition of CD1d-presented microbial antigens (Mattner et al., 2005; Tupin et al., 2007). Instead, our data suggest that innate cytokine-driven activation is the dominant pathway for iNKT cell activation in response to virtually all infectious agents examined to date that induce the production of IL-12 by APCs after TLR-mediated activation (Fig. 9).

In addition to IL-12 as a critical mediator for stimulating iNKT cell responses to microbes, other studies have suggested a role for IL-18 and type I IFN in the iNKT cell response to *E. coli* LPS or the TLR9 agonist CpG, respectively (Nagarajan and Kronenberg, 2007; Paget et al., 2007). However, our in vitro experiments did not show a significant role for IL-18 or type I IFNs in response to any of the 11 bacterial pathogens tested. Instead, our in vitro and in vivo experiments repeatedly implicated IL-12 as the critical cytokine required for

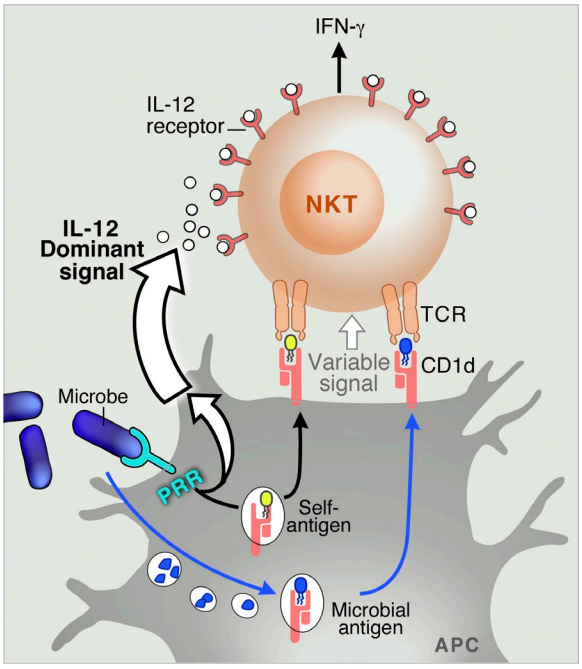

Figure 9. Innate and cytokine-driven iNKT cell activation during microbial infection. iNKT cell activation during microbial infection is dominantly driven by innate TLR-mediated signals and IL-12, which is released by DCs after stimulation with microbial products. In addition, TCR-mediated stimulation contributes to iNKT cell activation. However, the TCR-mediated signal alone, provided either by recognition of CD1d-presented self- or microbial antigens, is not sufficient to result in iNKT cell IFN-γ production in the absence of IL-12 stimulation. The constitutive expression of high levels of IL-12 receptor endows iNKT cells with the ability to respond rapidly to cytokine-mediated stimulation and ensures immediate iNKT cell activation in response to virtually any infectious agent that induces the production of IL-12, irrespective of the expression of microbial lipid antigens, thus allowing iNKT cells to overcome their restricted TCR specificity.

iNKT cell activation and IFN-γ secretion in response to all microbes tested. Underscoring the critical and dominant role of IL-12 for rapid activation of iNKT cells, we detected that constitutive expression of high levels of IL-12Rβ1 and IL-12Rβ2 chains by iNKT cells and IL-12 stimulation rapidly led to STAT4 activation in iNKT cells. In contrast, MHC-restricted T cells lack constitutive expression of components of the IL-12 receptor and are only known to express functional IL-12 receptor after activation and Th1 differentiation (Presky et al., 1996; Trinchieri, 2003). In agreement with previous studies, we found that naive and memory $CD4^+$ T cells were not able to activate STAT4 after IL-12 stimulation in the absence of additional stimulation or differentiation. Recently, MAIT (mucosal-associated invariant T) lymphocytes, another lymphocyte population characterized by expression of an invariant TCR-α chain, have been shown to become activated by microbes independently of TLR- and cytokine-mediated stimulation, suggesting that these innate T cells require recognition of cognate microbial antigens for their activation (Le Bourhis et al., 2010). Thus, among T cells, iNKT cells

appear to be uniquely equipped to promptly secrete IFN-γ upon primary encounter of an inflammatory condition that results in the production of IL-12.

Our in vitro experiments showed that full iNKT cell activation in response to all pathogens tested also required CD1d-dependent signals and that increased expression of CD1d by bacteria-stimulated APCs may contribute to iNKT cell activation. However, in the absence of co-stimulation by IL-12, the CD1d-mediated signals were not sufficient to induce IFN-γ secretion by iNKT cells. Our in vitro studies showed that iNKT cell responses to weak self-antigens or to low concentrations of microbial antigen were amplified by exogenously added IL-12. Therefore, although not able to activate iNKT cells through TCR-mediated signaling alone, it seems likely that even small numbers of CD1d–microbial antigen complexes or presentation of more potent self-antigens may contribute to or modulate iNKT cell activation during infection in the presence of IL-12–mediated co-stimulation. Furthermore, the requirement for CD1d-TCR–mediated signals may ensure that IL-12–amplified iNKT cell activation occurs only in close contact with CD1d-expressing APCs such as monocytes, DCs, macrophages, and B cells. The lack of dependence on specific recognition of CD1d-presented microbial antigens found in this study suggests that the exceptional potency of the pharmacological iNKT cell agonist α-GalCer, which is much greater than that of any naturally occurring iNKT cell antigen found to date, may have led to overestimation of the importance of microbial antigens in iNKT cell activation.

iNKT cell responses to all of the microbial antigens described so far result in the production of both IFN-γ and IL-4 by iNKT cells both in vitro and in vivo. Most of the bacteria tested in our studies resulted in a bias toward production of IFN-γ, using iNKT cell lines, freshly isolated iNKT cells, and during in vivo infection. Interestingly, the occasionally observed IL-4 induced by *Sphingomonas* spp. in vitro was increased in the absence of IL-12, suggesting that IL-12 may play an important role in polarizing iNKT cell responses to infection. However, in vivo infection with *Sphingomonas* spp. in IL-12–deficient mice did not result in a notable increase in the number of iNKT cells producing IL-4 (unpublished data). Stimulation of iNKT cells with *B. burgdorferi* resulted in IL-4 production by iNKT cells in our studies, which is consistent with previous studies detecting IL-4 production by a small number of iNKT cells in spleen after *B. burgdorferi* infection (Tupin et al., 2008). The in vitro IL-4 production observed in our studies appeared to be IL-12 independent but MyD88 dependent, suggesting that innate signals, rather than microbial antigens, are responsible for the iNKT cell IL-4 production in response to this bacterium. Further studies will be required to understand the nature of the signals responsible for bacteria-induced IL-4 production by iNKT cells.

Activation of iNKT cells during infection driven by innate signals and cytokines, rather than microbial antigens, provides several advantages. As an amplification loop for innate signals, cytokine-mediated activation enables the activation of iNKT cells in response to virtually any microorganism that induces IL-12 production, irrespective of the expression of iNKT cell ligands, and thus allows iNKT cells to overcome their restricted TCR repertoire and conserved mode of antigen-recognition. Such a mechanism of activation may be critical for iNKT cell responses to viral infection, because viruses are not known to encode enzymes for the production of unique microbial lipids, and for responses to other microorganisms that lack expression of CD1d-presented iNKT cell ligands. For the organisms that contain iNKT cell lipid antigens, synthesis, uptake, and loading of antigens into CD1d molecules, and subsequent transport of antigen–CD1d complexes to the surface of APCs, may be inefficient and slower than the IL-12–driven iNKT cell response.

The rapid innate cytokine-driven activation of iNKT cells provides the evolving immune response with T cell effector functions early during the course of infection that otherwise would only be available after the expansion and differentiation of antigen-specific MHC-restricted T cells (Brigl et al., 2003; Chiba et al., 2008). During infection, iNKT cell–secreted IFN-γ appears to serve several critically important functions. This includes a critical role in activation of macrophages and in neutrophil-mediated clearance of pathogens (Nieuwenhuis et al., 2002; Nakamatsu et al., 2007). In addition, the early IFN-γ provided by iNKT cells has been shown to be critical for the development of adaptive Th1 immune responses and may affect the magnitude and quality of MHC-restricted CD4 and CD8 T cell responses during infection (Tupin et al., 2007; Cohen et al., 2009). Together, iNKT cells, and the IFN-γ they produce, have emerged as critical regulators of the early immune response during infection.

In summary, our findings indicate that during infection with a variety of bacterial pathogens, including bacteria which express known iNKT cell antigens, iNKT cell activation is driven predominantly by IL-12, rather than by TCR-mediated recognition of CD1d-presented microbial antigens (Fig. 9). In contrast to the dominant role observed for foreign antigen recognition in the activation of MHC-restricted T cells, the lack of dependence on specific recognition of cognate microbial antigen defines a different role for the TCR in innate T cell activation. For the activation of iNKT cells during infection, CD1d–TCR interactions instead appear to determine the cell–cell interaction between iNKT cells and APCs and allow T cell activation to become manifest. This may control such innate cytokine-driven activation so that it is localized to sites of APC-produced IL-12. The constitutive expression of high levels of IL-12 receptor uniquely equips iNKT cell for immediate cytokine-driven responses. Our results suggest a unified dominantly innate cytokine-driven model of iNKT cell activation that explains how these cells become activated efficiently and rapidly in response to highly diverse microbial pathogens.

MATERIALS AND METHODS

Mice. C57BL/6, IL-12p40$^{-/-}$, IL-12p35$^{-/-}$, and IL-18$^{-/-}$ mice were obtained from The Jackson Laboratory. Jα18$^{-/-}$ (Cui et al., 1997) and CD1d$^{-/-}$ mice (Exley et al., 2003) were provided by M. Exley (Harvard Medical School, Boston, MA), Vα14Jα18 TCR transgenic mice (Bendelac et al., 1996)

by A. Bendelac (University of Chicago, Chicago, IL), and MyD88$^{-/-}$ (Adachi et al., 1998) by K. Kobayashi (Harvard Medical School, Boston, MA). All mice were on the C57BL/6 background, were housed in specific pathogen-free conditions or a biosafety level 2 animal facility after in vivo infections, and female mice at 6–14 wk of age were used for experiments. All animal studies were approved by the Dana-Faber Cancer Institute Animal Care and Use Committee.

In vitro iNKT cell assay. iNKT cell lines were generated and maintained in complete RPMI medium (RPMI supplemented with 10% FBS [Gemini], Hepes [Invitrogen], L-glutamine, penicillin/streptomycin, and 2-ME) supplemented with 20 U/ml of recombinant mouse IL-2 (PeproTech) and 10 ng/ml IL-7 as previously described (Chiba et al., 2009). DCs were generated from BM of mice cultured in complete RPMI supplemented with 10 ng/ml of recombinant mouse GM-CSF and 1 ng/ml IL-4 for 5–8 d and purified with CD11c magnetic beads (Miltenyi Biotec). iNKT cell lines (10^5/well) were cultured with DCs (2×10^4/well) in 96-well plates (Costar; Corning) in complete RPMI. Freshly isolated iNKT cells were obtained from spleens of WT B6 animals after depletion of CD19$^+$ and CD8a$^+$ cells using magnetic beads (Miltenyi Biotec), followed by cell sorting using CD1d tetramers and TCR-β staining on a FACSAria IIu instrument (BD). GSL-1 (National Institutes of Health [NIH] Tetramer Core Facility), LPS (*Salmonella enterica* serotype Typhimurium; Sigma-Aldrich), CpG (ODN1826; InvivoGen), recombinant mouse IL-12 (PeproTech), or bacteria were added and cultures were incubated for 16–48 h at 37°C. Culture supernatants were analyzed by ELISA with matched antibody pairs (IFN-γ and IL-4 [BD], sensitivity 10 and 2 pg/ml, respectively; IL-18 and IFN-β [R&D Systems]). Blocking anti–IL-12 (BD) and anti–mouse IFN-α/β R1 antibodies (R&D Systems) were added to cultures at 5–10 μg/ml. Expression of CD25 was determined on iNKT cells by flow cytometry (gated by FSC/SSC). Surface expression of CD1d on CD11c$^+$ BM DCs was determined using CD1d Abs (BD) after incubation with various stimuli.

Bacteria. *S. pneumoniae* (for strains and references see Table I; provided by K. Kawakami (University of the Ryukyus, Nishihara, Okinawa, Japan), cultured in THY [BD] or BHI [Oxoid] medium), *L. monocytogenes* (BHI medium), *S. aureus* (LB medium; BD), *P. aeruginosa* (LB medium, provided by R. Blumberg, Harvard Medical School, Boston, MA), *S. typhimurium* (LB medium), *E. coli* (LB medium), *S. capsulata* (MH medium, 30°C; Oxoid), *N. aromaticivorans* (MH medium), *S. yanoikuyae* (TSB), and *B. burgdorferi* (BSK-H medium, 33°C; provided by L. Bockenstedt, Yale School of Medicine, New Haven, CT; Sigma-Aldrich) were grown in culture medium at 37°C, unless otherwise noted, until mid- to late-log phase, washed 3× in LPS-free PBS (Invitrogen), and heat inactivated at 65°C for 45 min. *B. burgdorferi* were inactivated at 48°C for 30 min. *M. tuberculosis* were inactivated by γ radiation (Colorado State University, Fort Collins, CO). All bacterial preparations, except gram-negative bacteria, tested negative for LPS by Limulus amebocyte lysate test (limit of detection 0.03 EU/ml; Associates of Cape Cod).

Lipids, lipid extraction, and MS analysis. Lipids were extracted and analyzed by thin layer chromatography, as described elsewhere (Tatituri et al., 2007), or analyzed by high performance liquid chromatography (HPLC) using a AutoPurification HPLC system coupled to a mass detector with both ES and APCI capabilities, evaporative light scattering detector, and photodiode array detector (Waters). MS analysis, including low-energy CAD MSn experiments, were conducted on a linear ion-trap (LIT) mass spectrometer (Finnigan; Thermo Fisher Scientific) with Xcalibur operating system. Lipid extracts dissolved in chloroform/methanol (1/2) were continuously infused (2 μl/min) to the ESI source, where the skimmer was set at ground potential, the electrospray needle was set at 4.5 kV, and temperature of the heated capillary was 300°C. The automatic gain control of the ion trap was set to 2×10^4, with a maximum injection time of 100 ms. Helium was used as the buffer and collision gas at a pressure of 10^{-3} mbar (0.75 mTorr). The MSn experiments were performed with an optimized relative collision energy ranging from 18–25%, an activation q value at 0.25, and the activation time at 30–50 ms to leave a minimal residual abundance of precursor ion (around 10%). Mass spectra were accumulated in the profile mode, typically for 3–10 min for MSn (n = 2, 3, and 4) spectra. The mass resolution of the instrument was tuned to 0.6 D at half peak height. Synthetic GSL-1 was provided by the NIH tetramer facility and P.B. Savage (Brigham Young University, Provo, UT; Mattner et al., 2005). Synthesis of α-GalCer has been previously described (Veerapen et al., 2009).

In vitro infections. Bacteria were grown to mid-log phase, as described in Bacteria, and added to DCs cultured in antibiotic-free complete RPMI. After a 3-h incubation at 37°C, infected DCs were washed 3× in complete RPMI. Infected DCs (5×10^4/well) were co-cultured with iNKT cell lines (10^5/well) in complete RPMI medium in 96-well plates for 16–24 h.

In vivo infection and flow cytometry. *S. pneumoniae* was grown as described in Bacteria and mice were anesthetized with i.p. ketamine/xylazine or by inhalation isoflurane and inoculated intranasally or intratracheally, respectively, with $2–3 \times 10^6$ bacteria in PBS. CFUs were determined on blood agar plates. For flow cytometric analysis, mononuclear cells were isolated from lungs perfused with PBS followed by digestion with collagenase/DNase. *S. yanoikuyae* was grown in TSB supplemented with 5% sheep blood and passaged in SCID mice. Mice were injected i.v. with 4×10^9 bacteria in 200 μl PBS. Cells were stained with antibodies against CD45, CD19, TCR-β, PBS57-loaded CD1d tetramers (NIH Tetramer Core Facility), CD69, or isotype-matched control antibodies. Secretion of IFN-γ or IL-4 was determined by cytokine secretion assay (Miltenyi Biotec) according to the manufacturer's instructions. Flow cytometric data were collected on a LSR II flow cytometer (BD) and analyzed with FlowJo software (Tree Star).

IL-12 receptor expression. iNKT cells were purified from spleen or liver on a MoFlo cell sorter (Dako) gating on CD19$^-$, B220$^-$, Ter119$^-$, CD11b$^-$, CD11c$^-$, LyG/Gr-1$^-$, CD45$^+$, TCR^{int}, CD1d-tetramer$^+$, CD4$^+$, or CD4$^-$ iNKT cells, followed by RNA extraction with Trizol (Invitrogen), RNA amplified, and hybridized to Affymetrix 1.0 ST MuGene arrays within the NIAID/NIH ImmGen project (protocols and raw data available at http://www.immgen.org; Heng and Painter, 2008). For flow cytometric analysis, cells were stained with CD1d tetramers and antibodies against IL-12Rβ1 (BD).

STAT4 phosphorylation. Naive (CD62L^{hi}CD44^{lo}) and memory (CD62L^{lo}CD44^{hi}) TCRβ$^+$CD4$^+$ T cells, NK cells (NK1.1$^+$TCR-β$^-$), and iNKT cells (as in the previous section) were isolated by negative selection and cell sorting. $1–5 \times 10^5$ purified cells were cultured in completed RPMI and stimulated with recombinant IL-12 for 1 h at 37°C and subsequently stained with antibodies against pSTAT4 (BD) according to manufacturer's protocols.

Online supplemental material. Fig. S1 shows mass spectrometry analysis of microbial lipids separated by liquid chromatography. Fig. S2 shows representative dose titrations for bar graphs depicted in Fig. 4 (A and C). Fig. S3 shows IL-18 and type I IFN independence of iNKT cell activation in response to microbes. Fig. S4 shows representative FACS plots for bar graphs depicted in Fig. 4 D. Fig. S5 shows IL-12 dependence of IFN-γ secretion and CD25 expression by iNKT cells on day 2 after activation with microbial products. Fig. S6 shows cytokine responses to microbial stimulation of freshly isolated iNKT cells. Fig. S7 shows MyD88-dependent CD1d expression by DCs after stimulation with microbial products. Fig. S8 shows survival of WT and CD1d-deficient mice after *S. pneumoniae* infection and in vivo activation of iNKT cells on day 4 after *S. pneumoniae* infection. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20102555/DC1.

The authors would like to thank the NIH Tetramer Core Facility for mouse CD1d tetramer and GSL-1, A. Bendelac, M. Exley, and K. Kobayashi for providing mice, K. Kawakami, L. Bockenstedt, and R. Blumberg for providing bacteria, P.B. Savage for providing GSL-1, P. Brennan for critically reading the manuscript, and S. Moskowitz for help with the artwork.

This work was supported by NIH grants AI077795 (M. Brigl), AI028973, and AI063428 (both M.B. Brenner) and grants P41-RR-00954, R37-DK-34388, and P60-DK-20579 (Mass Spectrometry Center, Washington University).

The authors have no conflicting financial interests.

Submitted: 8 December 2010
Accepted: 1 April 2011

REFERENCES

Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity*. 9:143–150. doi:10.1016/S1074-7613(00)80596-8

Arrunategui-Correa, V., and H.S. Kim. 2004. The role of CD1d in the immune response against *Listeria* infection. *Cell. Immunol.* 227:109–120. doi:10.1016/j.cellimm.2004.02.003

Behar, S.M., C.C. Dascher, M.J. Grusby, C.R. Wang, and M.B. Brenner. 1999. Susceptibility of mice deficient in CD1D or TAP1 to infection with *Mycobacterium tuberculosis. J. Exp. Med.* 189:1973–1980. doi:10.1084/jem.189.12.1973

Bendelac, A., R.D. Hunziker, and O. Lantz. 1996. Increased interleukin 4 and immunoglobulin E production in transgenic mice overexpressing NK1 T cells. *J. Exp. Med.* 184:1285–1293. doi:10.1084/jem.184.4.1285

Bendelac, A., P.B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu. Rev. Immunol.* 25:297–336. doi:10.1146/annurev.immunol.25.022106.141711

Berntman, E., J. Rolf, C. Johansson, P. Anderson, and S.L. Cardell. 2005. The role of CD1d-restricted NK T lymphocytes in the immune response to oral infection with *Salmonella typhimurium*. *Eur. J. Immunol.* 35:2100–2109. doi:10.1002/eji.200425846

Brigl, M., and M.B. Brenner. 2004. CD1: antigen presentation and T cell function. *Annu. Rev. Immunol.* 22:817–890. doi:10.1146/annurev.immunol.22.012703.104608

Brigl, M., and M.B. Brenner. 2010. How invariant natural killer T cells respond to infection by recognizing microbial or endogenous lipid antigens. *Semin. Immunol.* 22:79–86. doi:10.1016/j.smim.2009.10.006

Brigl, M., L. Bry, S.C. Kent, J.E. Gumperz, and M.B. Brenner. 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat. Immunol.* 4:1230–1237. doi:10.1038/ni1002

Burrows, P.D., M. Kronenberg, and M. Taniguchi. 2009. NKT cells turn ten. *Nat. Immunol.* 10:669–671. doi:10.1038/ni0709-669

Chiba, A., C.C. Dascher, G.S. Besra, and M.B. Brenner. 2008. Rapid NKT cell responses are self-terminating during the course of microbial infection. *J. Immunol.* 181:2292–2302.

Chiba, A., N. Cohen, M. Brigl, P.J. Brennan, G.S. Besra, and M.B. Brenner. 2009. Rapid and reliable generation of invariant natural killer T-cell lines in vitro. *Immunology*. 128:324–333. doi:10.1111/j.1365-2567.2009.03130.x

Cohen, N.R., S. Garg, and M.B. Brenner. 2009. Antigen Presentation by CD1 Lipids, T Cells, and NKT Cells in Microbial Immunity. *Adv. Immunol.* 102:1–94. doi:10.1016/S0065-2776(09)01201-2

Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science*. 278:1623–1626. doi:10.1126/science.278.5343.1623

Exley, M.A., N.J. Bigley, O. Cheng, A. Shaulov, S.M. Tahir, Q.L. Carter, J. Garcia, C. Wang, K. Patten, H.F. Stills, et al. 2003. Innate immune response to encephalomyocarditis virus infection mediated by CD1d. *Immunology*. 110:519–526. doi:10.1111/j.1365-2567.2003.01779.x

Fischer, K., E. Scotet, M. Niemeyer, H. Koebernick, J. Zerrahn, S. Maillet, R. Hurwitz, M. Kursar, M. Bonneville, S.H. Kaufmann, and U.E. Schaible. 2004. Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. *Proc. Natl. Acad. Sci. USA*. 101:10685–10690. doi:10.1073/pnas.0403787101

Godfrey, D.I., H.R. MacDonald, M. Kronenberg, M.J. Smyth, and L. Van Kaer. 2004. NKT cells: what's in a name? *Nat. Rev. Immunol.* 4:231–237. doi:10.1038/nri1309

Heng, T.S., and M.W. Painter; Immunological Genome Project Consortium. 2008. The Immunological Genome Project: networks of gene expression in immune cells. *Nat. Immunol.* 9:1091–1094. doi:10.1038/ni1008-1091

Kawakami, K., Y. Kinjo, S. Yara, K. Uezu, Y. Koguchi, M. Tohyama, M. Azuma, K. Takeda, S. Akira, and A. Saito. 2001. Enhanced gamma interferon production through activation of Valpha14(+) natural killer T cells by alpha-galactosylceramide in interleukin-18-deficient mice with systemic cryptococcosis. *Infect. Immun.* 69:6643–6650. doi:10.1128/IAI.69.11.6643-6650.2001

Kawakami, K., N. Yamamoto, Y. Kinjo, K. Miyagi, C. Nakasone, K. Uezu, T. Kinjo, T. Nakayama, M. Taniguchi, and A. Saito. 2003. Critical role of Valpha14$^+$ natural killer T cells in the innate phase of host protection against *Streptococcus pneumoniae* infection. *Eur. J. Immunol.* 33:3322–3330. doi:10.1002/eji.200324254

Kinjo, Y., D. Wu, G. Kim, G.W. Xing, M.A. Poles, D.D. Ho, M. Tsuji, K. Kawahara, C.H. Wong, and M. Kronenberg. 2005. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature*. 434:520–525. doi:10.1038/nature03407

Kinjo, Y., E. Tupin, D. Wu, M. Fujio, R. Garcia-Navarro, M.R. Benhnia, D.M. Zajonc, G. Ben-Menachem, G.D. Ainge, G.F. Painter, et al. 2006. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat. Immunol.* 7:978–986. doi:10.1038/ni1380

Kitamura, H., K. Iwakabe, T. Yahata, S. Nishimura, A. Ohta, Y. Ohmi, M. Sato, K. Takeda, K. Okumura, L. Van Kaer, et al. 1999. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J. Exp. Med.* 189:1121–1128. doi:10.1084/jem.189.7.1121

Kronenberg, M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu. Rev. Immunol.* 23:877–900. doi:10.1146/annurev.immunol.23.021704.115742

Kumar, H., A. Belperron, S.W. Barthold, and L.K. Bockenstedt. 2000. Cutting edge: CD1d deficiency impairs murine host defense against the spirochete, *Borrelia burgdorferi*. *J. Immunol.* 165:4797–4801.

Le Bourhis, L., E. Martin, I. Péguillet, A. Guihot, N. Froux, M. Coré, E. Lévy, M. Dusseaux, V. Meyssonnier, V. Premel, et al. 2010. Antimicrobial activity of mucosal-associated invariant T cells. *Nat. Immunol.* 11:701–708. doi:10.1038/ni.1890

Matsuda, J.L., L. Gapin, J.L. Baron, S. Sidobre, D.B. Stetson, M. Mohrs, R.M. Locksley, and M. Kronenberg. 2003. Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. *Proc. Natl. Acad. Sci. USA*. 100:8395–8400. doi:10.1073/pnas.1332805100

Mattner, J., K.L. Debord, N. Ismail, R.D. Goff, C. Cantu III, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, et al. 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature*. 434:525–529. doi:10.1038/nature03408

Mattner, J., P.B. Savage, P. Leung, S.S. Oertelt, V. Wang, O. Trivedi, S.T. Scanlon, K. Pendem, L. Teyton, J. Hart, et al. 2008. Liver autoimmunity triggered by microbial activation of natural killer T cells. *Cell Host Microbe*. 3:304–315. doi:10.1016/j.chom.2008.03.009

Mohammed, J.P., and J. Mattner. 2009. Autoimmune disease triggered by infection with alphaproteobacteria. *Expert Rev. Clin. Immunol.* 5:369–379. doi:10.1586/eci.09.23

Nagarajan, N.A., and M. Kronenberg. 2007. Invariant NKT cells amplify the innate immune response to lipopolysaccharide. *J. Immunol.* 178:2706–2713.

Nakamatsu, M., N. Yamamoto, M. Hatta, C. Nakasone, T. Kinjo, K. Miyagi, K. Uezu, K. Nakamura, T. Nakayama, M. Taniguchi, et al. 2007. Role of interferon-gamma in Valpha14+ natural killer T cell-mediated host defense against *Streptococcus pneumoniae* infection in murine lungs. *Microbes Infect.* 9:364–374. doi:10.1016/j.micinf.2006.12.003

Nieuwenhuis, E.E., T. Matsumoto, M. Exley, R.A. Schleipman, J. Glickman, D.T. Bailey, N. Corazza, S.P. Colgan, A.B. Onderdonk, and R.S. Blumberg. 2002. CD1d-dependent macrophage-mediated clearance of *Pseudomonas aeruginosa* from lung. *Nat. Med.* 8:588–593. doi:10.1038/nm0602-588

Paget, C., T. Mallevaey, A.O. Speak, D. Torres, J. Fontaine, K.C. Sheehan, M. Capron, B. Ryffel, C. Faveeuw, M. Leite de Moraes, et al. 2007. Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. *Immunity*. 27:597–609. doi:10.1016/j.immuni.2007.08.017

Presky, D.H., H. Yang, L.J. Minetti, A.O. Chua, N. Nabavi, C.Y. Wu, M.K. Gately, and U. Gubler. 1996. A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. *Proc. Natl. Acad. Sci. USA*. 93:14002–14007. doi:10.1073/pnas.93.24.14002

Raghuraman, G., Y. Geng, and C.R. Wang. 2006. IFN-beta-mediated upregulation of CD1d in bacteria-infected APCs. *J. Immunol.* 177:7841–7848.

Ryan, M.P., and C.C. Adley. 2010. *Sphingomonas paucimobilis*: a persistent Gram-negative nosocomial infectious organism. *J. Hosp. Infect.* 75:153–157. doi:10.1016/j.jhin.2010.03.007

Salio, M., A.O. Speak, D. Shepherd, P. Polzella, P.A. Illarionov, N. Veerapen, G.S. Besra, F.M. Platt, and V. Cerundolo. 2007. Modulation of human natural killer T cell ligands on TLR-mediated antigen-presenting cell activation. *Proc. Natl. Acad. Sci. USA*. 104:20490–20495. doi:10.1073/pnas.0710145104

Sköld, M., X. Xiong, P.A. Illarionov, G.S. Besra, and S.M. Behar. 2005. Interplay of cytokines and microbial signals in regulation of CD1d expression and NKT cell activation. *J. Immunol.* 175:3584–3593.

Sriram, V., W. Du, J. Gervay-Hague, and R.R. Brutkiewicz. 2005. Cell wall glycosphingolipids of *Sphingomonas paucimobilis* are CD1d-specific ligands for NKT cells. *Eur. J. Immunol.* 35:1692–1701. doi:10.1002/eji.200526157

Stetson, D.B., M. Mohrs, R.L. Reinhardt, J.L. Baron, Z.E. Wang, L. Gapin, M. Kronenberg, and R.M. Locksley. 2003. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J. Exp. Med.* 198:1069–1076. doi:10.1084/jem.20030630

Tatituri, R.V., P.A. Illarionov, L.G. Dover, J. Nigou, M. Gilleron, P. Hitchen, K. Krumbach, H.R. Morris, N. Spencer, A. Dell, et al. 2007. Inactivation of *Corynebacterium glutamicum* NCgl0452 and the role of MgtA in the biosynthesis of a novel mannosylated glycolipid involved in lipomannan biosynthesis. *J. Biol. Chem.* 282:4561–4572. doi:10.1074/jbc.M608695200

Tomura, M., W.G. Yu, H.J. Ahn, M. Yamashita, Y.F. Yang, S. Ono, T. Hamaoka, T. Kawano, M. Taniguchi, Y. Koezuka, and H. Fujiwara. 1999. A novel function of Valpha14+CD4+ NKT cells: stimulation of IL-12 production by antigen-presenting cells in the innate immune system. *J. Immunol.* 163:93–101.

Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3:133–146. doi:10.1038/nri1001

Tupin, E., Y. Kinjo, and M. Kronenberg. 2007. The unique role of natural killer T cells in the response to microorganisms. *Nat. Rev. Microbiol.* 5:405–417. doi:10.1038/nrmicro1657

Tupin, E., M.R. Benhnia, Y. Kinjo, R. Patsey, C.J. Lena, M.C. Haller, M.J. Caimano, M. Imamura, C.H. Wong, S. Crotty, et al. 2008. NKT cells prevent chronic joint inflammation after infection with *Borrelia burgdorferi*. *Proc. Natl. Acad. Sci. USA*. 105:19863–19868. doi:10.1073/pnas.0810519105

Tyznik, A.J., E. Tupin, N.A. Nagarajan, M.J. Her, C.A. Benedict, and M. Kronenberg. 2008. Cutting edge: the mechanism of invariant NKT cell responses to viral danger signals. *J. Immunol.* 181:4452–4456.

Veerapen, N., M. Brigl, S. Garg, V. Cerundolo, L.R. Cox, M.B. Brenner, and G.S. Besra. 2009. Synthesis and biological activity of alpha-galactosyl ceramide KRN7000 and galactosyl (alpha1—>2) galactosyl ceramide. *Bioorg. Med. Chem. Lett.* 19:4288–4291. doi:10.1016/j.bmcl.2009.05.095

Wesley, J.D., M.S. Tessmer, D. Chaukos, and L. Brossay. 2008. NK cell-like behavior of Valpha14i NK T cells during MCMV infection. *PLoS Pathog.* 4:e1000106. doi:10.1371/journal.ppat.1000106