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Invariant NKT Cells Amplify the Innate Immune Response to Lipopolysaccharide¹

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NKT cells are thought of as a bridge between innate and adaptive immunity. In this study, we demonstrate that mouse NKT cells are activated in response to *Escherichia coli* LPS, and produce IFN- γ , but not IL-4, although activation through their TCR typically induces both IL-4 and IFN- γ production. IFN- γ production by NKT cells is dependent on LPS-induced IL-12 and IL-18 from APC. LPS induced IFN- γ production by NKT cells does not require CD1d-mediated presentation of an endogenous Ag and exposure to a combination of IL-12 and IL-18 is sufficient to activate them. In mice that are deficient for NKT cells, innate immune cells are activated less efficiently in response to LPS, resulting in the reduced production of TNF and IFN- γ . We propose that in addition to acting as a bridge to adaptive immunity, NKT cells act as an early amplification step in the innate immune response and that the rapid and complete initiation of this innate response depends on the early production of IFN- γ by NKT cells. *The Journal of Immunology*, 2007, 178: 2706–2713.

Natural killer T cells were originally defined as T lymphocytes that express NK1.1 and other receptors typical of NK cells. This definition has since been narrowed in many cases to specify a subpopulation of T lymphocytes that express a TCR with an invariant V α 14-J α 18 TCR α chain paired with a restricted subset of TCR V β chains (1). These cells are also called invariant NKT (iNKT)³ cells, or V α 14i NKT cells, and they recognize glycosphingolipid (GSL) Ags presented by CD1d, a nonclassical class I-like Ag-presenting molecule. V α 14i NKT cells respond rapidly to TCR stimulation with a model GSL Ag, α -galactosylceramide (α GalCer), resulting in the synthesis of many cytokines, including IFN- γ and IL-4 simultaneously (1). However, in many disease models where V α 14i NKT cells have been shown to be important, it is usually the action of either Th1 or Th2 cytokines that is important, rarely both simultaneously (2). In particular, although there are exceptions, IFN- γ production by V α 14i NKT cells has been implicated in the clearance of many infections, while IL-4 production appears to be necessary to ameliorate some autoimmune and inflammatory conditions (2).

Recent work has shown that the AgR expressed by V α 14i NKT cells recognizes GSL Ags from *Sphingomonas* bacteria (3–5) and diacyl glycerol Ags from *Borrelia burgdorferi* (6), presented by CD1d. This so-called “direct” recognition is important for the early

stages of bacterial clearance (3, 4). The identification of these bacterial Ags does not provide an explanation for the role of V α 14i NKT cells in all the models of bacterial infection in which they have been implicated. There must be means, therefore, by which V α 14i NKT cells recognize microbes that do not have an Ag for the invariant TCR.

The response to LPS, long considered a paradigm for innate immunity, provides a model system for understanding how V α 14i NKT cells may be able to respond to microbes in the absence of TCR engagement by a foreign Ag. LPS, a component of the cell wall of many Gram-negative bacteria, signals through a complex consisting of CD14, TLR4, and MD-2 (7), leading to the secretion of proinflammatory cytokines and potent activation of the innate immune system (8). The purpose of this proinflammatory cascade is to facilitate bacterial clearance, but it can have deleterious effects such as septic shock (9) when induced to excess. IFN- γ derived from V α 14i NKT cells has been proposed to be one of the critical effectors of the Shwartzman reaction, an animal model of septic shock induced by LPS (10, 11), but these data are controversial (12). The mechanism by which V α 14i NKT cells produce IFN- γ in response to LPS has not been entirely determined. There have been studies that report TLR expression by these T cells (13–15), however, there have been others that demonstrate a so-called “indirect” pathway by which V α 14i NKT cells are activated in response to *Salmonella* or *Salmonella* LPS (4, 16). The indirect pathway postulates that V α 14i NKT cells respond to LPS by integrating two types of signals: one signal is IL-12 produced by activated APCs, which stimulates the IL-12R expressed by V α 14i NKT cells, and the other is the recognition of a relatively weak endogenous GSL Ag presented by CD1d, which stimulates the V α 14i TCR (16).

In this study, we describe another pathway, dependent only on APC-derived cytokines, which constitutes a third means by which V α 14i NKT cells can respond to bacteria. Additionally, we show that the systemic response to LPS is severely impaired in the absence of V α 14i NKT cells, indicating that in addition to bridging innate and adaptive immunity, V α 14i NKT cells can act rapidly to amplify innate immune responses initiated by APC, even in the absence of a TCR-mediated signal.

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³ Abbreviations used in this paper: iNKT, invariant NKT; GSL, glycosphingolipid; α GalCer, α -galactosylceramide; rm, recombinant murine; ICCS, intracellular cytokine staining; DC, dendritic cell; Tg, transgenic; BM, bone marrow.

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Materials and Methods

Mice

C57BL/6J, BALB/cJ, B6.129S1-*Il12b*^{tm1Jm}/J (*IL-12p40*^{-/-}), and B6.129P2-*Il18*^{tm1Aki}/J (*IL-18*^{-/-}) mice were obtained from The Jackson Laboratory and maintained in the vivarium at the La Jolla Institute for Allergy and Immunology (La Jolla, CA). *CD1d*^{-/-} mice on the C57BL/6J background were a gift from Dr. L. Van Kaer (Vanderbilt University, Nashville, TN). B6.129-*Tcra*-*Jm1Tg* (*Ja18*^{-/-}) mice on the C57BL/6J background were a gift from Dr. M. Taniguchi (RIKEN Institute, Yokohama, Japan). Vα14-Jα18 transgenic mice on the C57BL/6J.Cα^{-/-} background were a gift from Dr. A. Bendelac (University of Chicago, Chicago, IL). Unless otherwise mentioned, all mice were used between 8 and 16 wk of age, and were age and gender matched.

Reagents and Abs

Escherichia coli O111:B4 LPS and *Salmonella abortus equi* LPS were purchased from Sigma-Aldrich, either purified by phenol extraction (used for injections) or phenol extracted and further purified by ion-exchange chromatography (used for cell culture).

Abs to the following mouse Ags were purchased from BD Biosciences, as conjugates to FITC, Alexa 488, PE, PerCP-Cy5.5, PE-Cy7, allophycocyanin, or biotin: CD1d, CD4, CD8α, CD11b, CD11c, CD18, CD69, Gr-1, NK1.1, TCRβ, IFN-γ, and TNF. Allophycocyanin-conjugated anti-F4/80 was purchased from eBioscience. Anti-mouse IL-4-Alexa 488 was obtained from Caltag Laboratories. Anti-mouse macrophage (CD68) was purchased from Serotec. Anti-mouse CD16/32 used for FcR blocking was isolated in our laboratory. Purified anti-mouse CD1d (1B1) for blocking experiments was obtained from BD Biosciences. PE-conjugated αGalCer-CD1d tetramers were synthesized in our laboratory as described (17), and used to stain cell suspensions. Recombinant mouse GM-CSF (rmGM-CSF) was purchased from PeproTech. Recombinant mouse IL-12 and IL-18 (rmIL-12 and rmIL-18) were purchased from R&D Systems. α-Galactosylceramide (αGalCer; (2S,3S,4R)-1-O-(α-D-galactopyranosyl)-N-hexacosanoyl-2-amino-1,3,4-octadecanetriol) was synthesized at Kirin Pharmaceuticals (18, 19).

Treatments

All treatment protocols were conducted according to Institutional animal care guidelines. Mice were injected i.v. in the tail vein in a final volume of 200 μl, and blood was harvested by cardiac puncture after sacrifice. Mice were injected with 40 μg of LPS diluted in PBS, and 2 μg of αGalCer in vehicle, unless otherwise mentioned.

Cell preparation

All cell preparations were conducted using complete RPMI (RPMI 1640; Invitrogen Life Technologies) supplemented with 10% FCS, penicillin-streptomycin-glutamine, and 2-ME. Livers were perfused with ice-cold PBS before isolation of myeloid cells. Livers were mashed through a 70-mm cell strainer (Fisher Scientific) and washed. The suspension was centrifuged over a 40–70% Percoll (Amersham Biosciences) gradient and mononuclear cells were isolated by harvesting the interphase. Liver mononuclear cells were resuspended in the appropriate amount of medium and stained before FACS or sorting. Spleens were mashed and RBC were lysed using red cell lysing buffer from Sigma-Aldrich, after which the cells were resuspended in complete RPMI 1640. The cell suspension was stained and used for FACS, sorting, or for cell culture.

Staining for flow cytometry and intracellular cytokine staining

Cells were resuspended in staining buffer (PBS with 10% FCS) and anti-CD16/32 was added to block nonspecific staining. Abs to surface markers were added and cells were stained for 20 min on ice. Cells were washed and resuspended in staining buffer and analyzed on a FACSCalibur (BD Biosciences).

Cell suspensions obtained from treated mice were stained for surface markers as described above, then fixed and permeabilized using CytoFix/CytoPerm buffer from BD Biosciences, according to the manufacturer's protocol. Abs against intracellular cytokines or CD68 were added and the cells were stained on ice for 30 min. Cells were washed and resuspended in staining buffer and analyzed on a FACSCalibur.

FACS sorting and cell purification

Vα14i NKT cells were sorted from livers or spleens, using labeling with αGalCer-CD1d tetramers and anti-TCRβ, and NKT cells were sorted with anti-NK1.1 and either anti-TCRβ or anti-CD5. Prior to sorting with CD1d-αGalCer tetramers, spleen cells were depleted of B cells and CD8 T cells

using anti-CD19 and anti-CD8α conjugated magnetic beads and MACS columns (Miltenyi Biotec), according to the manufacturer's protocol. The CD19⁻CD8⁻ cell suspension was labeled with αGalCer-CD1d tetramers and anti-TCRβ mAb, washed, and sorted. For sorting NK1.1⁺TCRβ⁺ or NK1.1⁺CD5⁺ cells, spleen suspensions were labeled with anti-NK1.1-PE and anti-TCRβ or anti-CD5 Abs and NK1.1⁺ cells were enriched using anti-PE-conjugated magnetic MACS beads (Miltenyi Biotec), washed, and sorted. CD8α⁺ and CD11b⁺ cells were rigorously excluded while sorting for NK1.1⁺TCRβ⁺ or NK1.1⁺CD5⁺ cells. Cells were routinely sorted to >97% purity on a FACSDiva (BD Biosciences). For the enrichment of Vα14i NKT cells from Vα14-transgenic (Tg) mice, spleen cells from these mice were first depleted of CD8α⁺ and CD19⁺ cells using MACS beads as described above. The depleted fraction was then further depleted of residual CD8α⁺, CD19⁺, B220⁺, CD11c⁺, CD11b⁺, and TCRγδ⁺ cells by labeling with biotinylated Abs to the listed Ags (eBioscience) followed by addition of anti-biotin conjugated MACS beads. The percentage of Vα14i NKT cells in the enriched fraction was determined and the equivalent of 5 × 10⁴ Vα14i NKT cells was cultured with bone marrow (BM) dendritic cells (DCs) as described below.

BM-derived DCs and cell culture

BM DCs were prepared as described (20). Briefly, BM was harvested from femurs of mice and cultured in 100-mm bacterial dishes in 10 ml of complete RPMI with 200 U/ml rmGM-CSF. On day 3 of the culture, 10 ml more of complete RPMI with 200 U/ml rmGM-CSF were added. BM DCs were harvested on day 6 and cultured with sorted NKT cells, with the addition of LPS or recombinant cytokines. A total of 2.5 × 10⁵ BM DCs were cultured with 5 × 10⁴ sorted NKT cells in a round-bottom 96-well plate at 37°C and 5% CO₂ for 48 h, as described (4). The culture supernatants were harvested, and IFN-γ and IL-4 levels measured by ELISA.

Cytokine detection

Abs and recombinant cytokine standards for IFN-γ, IL-4, and IL-12 ELISAs were purchased from BD Biosciences. IL-18 and TNF ELISA kits were purchased from R&D Systems. All ELISAs were conducted according to the manufacturer's instructions. Cytokine estimation by cytometric bead array was conducted using the BioPlex system from Bio-Rad and results were read using a Luminex plate reader.

RNA preparation and real-time PCR

Cells were resuspended in TRIzol (Invitrogen Life Technologies) for RNA isolation. Proteins and DNA removed by precipitation with chloroform and RNA was extracted by precipitation with isopropanol. The resulting total RNA was used for cDNA synthesis by the Superscript II system (Invitrogen Life Technologies). cDNA was used for real-time PCR using SYBR green (Bio-Rad), with the following primers: IFN-γ forward, 5'-GGATGCATTCATGAGTATTGC, reverse, 5'-CCTTTTCCGCTTCCTGAGG; TNF forward, 5'-AACTAGTGGTGCCAGCCGAT, reverse, 5'-CTTCA CAGAGCAATGACTCC. Gene expression was normalized to L32, forward, 5'-GAAACTGGCGGAAACCCA and reverse, 5'-GGATCTGG CCCTTGAACCTT. Data was collected and analyzed on an ABI GeneAmp5700 (Applied Biosystems).

Statistical analysis

Statistical analysis was conducted using the GraphPad Prism software package from GraphPad Software.

Results

Vα14i NKT cells produce exclusively IFN-γ after stimulation with LPS

It has been shown that Vα14i NKT cells produce IFN-γ after exposure to LPS (11, 16). To determine whether they produce other cytokines, mice were injected i.v. with 40 μg of *E. coli* LPS and cytokine production by Vα14i NKT cells was assayed by intracellular cytokine staining (ICCS) of gated αGalCer-CD1d tetramer⁺ cells, analyzed directly ex vivo, without further stimulation or addition of brefeldin A. Vα14i NKT cells showed increased expression of the early activation Ag CD69 in response to LPS as early as 2 h after injections (data not shown). However, production of IFN-γ by Vα14i NKT cells from the liver and the spleen was detected by ICCS only 3 h after LPS injection (data not shown) and peaked at 6 h (Fig. 1A), with ~30% of the cells positive.

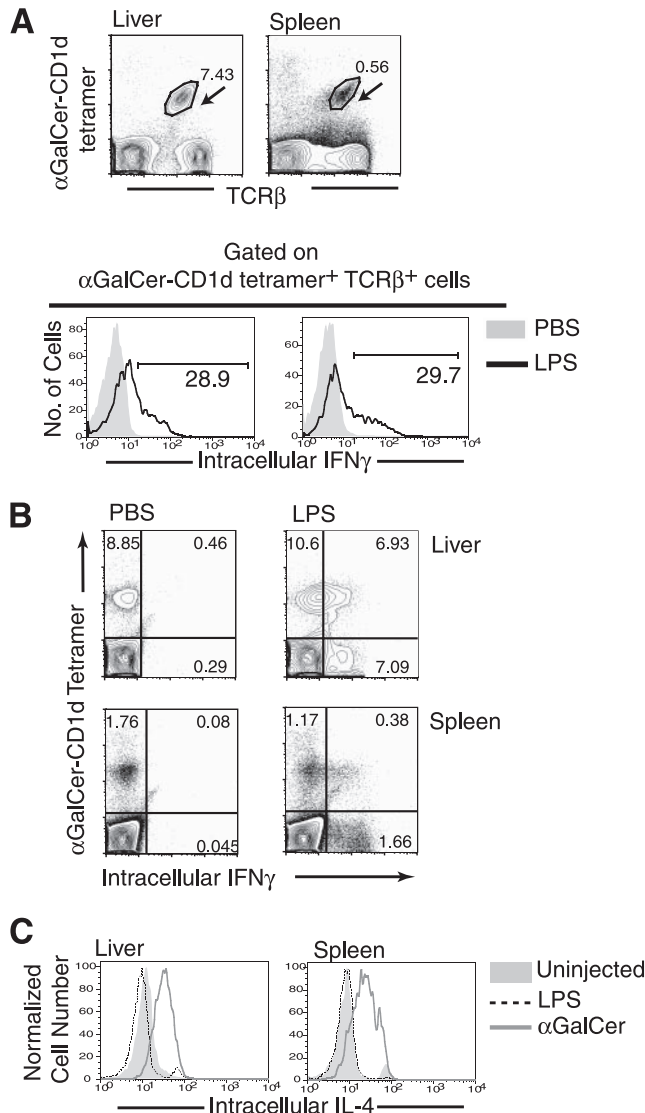


FIGURE 1. $V\alpha 14i$ NKT cells produce IFN- γ but no IL-4 in response to LPS. **A** and **B**, $V\alpha 14i$ NKT cells in both the livers and spleens of C57BL/6J mice produced IFN- γ as measured by ICCS 6 h after LPS injection. **A**, IFN- γ production specifically by $V\alpha 14i$ NKT cells. The numbers indicate the percentage of gated $V\alpha 14i$ NKT cells producing IFN- γ . **Top panels**, The gating for $V\alpha 14i$ NKT cells (arrows). **B**, IFN- γ production compared between $V\alpha 14i$ NKT cells and other cells in the spleen and liver. Numbers indicate percentage of total live cells in the quadrant. Data shown are from one mouse of a group of two, each experiment was repeated at least three times. **C**, $V\alpha 14i$ NKT cells do not produce IL-4 as measured by ICCS 6 h after LPS injection, but do produce IL-4 1.5 h after α GalCer injection. Uninjected (shaded histograms) and LPS injected (dotted lines) are essentially superimposable. One representative experiment of at least three.

IFN- γ production did not correlate with CD4 expression or any other cell surface marker tested (data not shown). At this time point after LPS injection, $V\alpha 14i$ NKT cells comprised approximately half of the total IFN- γ -producing cells in the liver and one-fifth of those in the spleen (Fig. 1B). The other IFN- γ -producing cells at this time were mostly NK cells (data not shown). Strikingly, $V\alpha 14i$ NKT cells did not produce detectable IL-4 (Fig. 1C), in contrast to the outcome following stimulation with α GalCer (Fig. 1C). $V\alpha 14i$ NKT cells also did not produce detectable TNF between 1 and 6 h after LPS injection, although this cytokine also is induced by α GalCer-mediated TCR stimulation (data not shown). These data show that $V\alpha 14i$ NKT cells produce

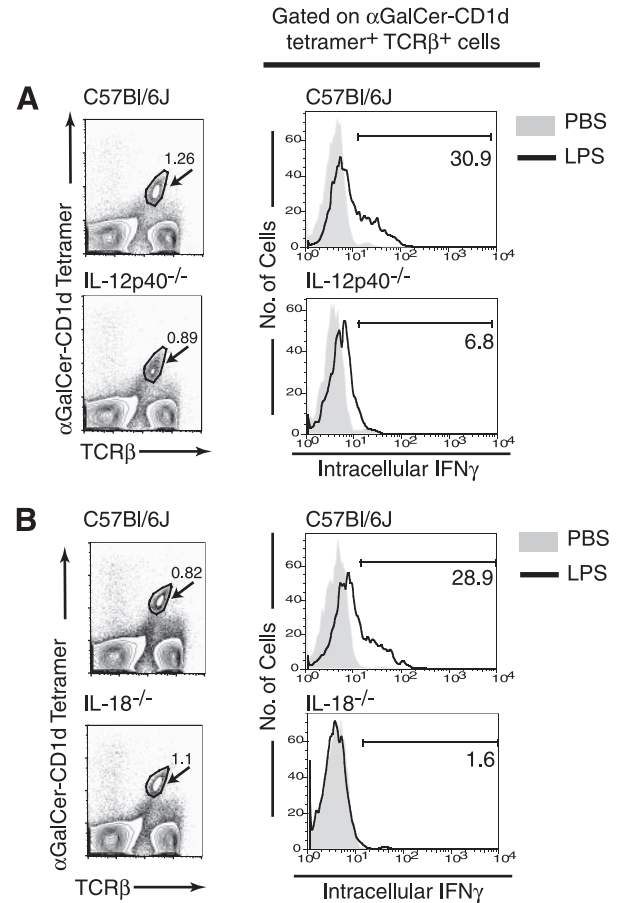


FIGURE 2. IFN- γ production by $V\alpha 14i$ NKT cells in response to LPS depends on IL-12 and IL-18. Mice deficient in the p40 subunit of IL-12 (**A**) or IL-18 were injected with LPS (**B**) and IFN- γ production by spleen $V\alpha 14i$ NKT cells was assayed using ICCS 6 h later. Panels on the left show the α GalCer-CD1d tetramer $^{+}$ population that was gated (arrows). Data shown are from one mouse of a group of two, each experiment was repeated three times.

a more limited set of cytokines after LPS stimulation compared with GSL Ag stimulation.

V $\alpha 14i$ NKT cells do not respond directly to LPS

To establish the mechanism by which $V\alpha 14i$ NKT cells responded to LPS, α GalCer-CD1d tetramer $^{+}$ cells were stained with Abs to the TLR4-MD2 complex to determine whether these cells could directly recognize LPS. We did not detect the presence of this complex on the surface of $V\alpha 14i$ NKT cells (data not shown). Furthermore, real-time PCR performed on $V\alpha 14i$ NKT cells FACS sorted from spleen (data not shown) did not detect TLR4 mRNA.

Studies with *Salmonella* LPS demonstrated that $V\alpha 14i$ NKT cells require IL-12 production by APCs to respond to LPS (16). In accordance with the published results, $V\alpha 14i$ NKT cells from *IL-12p40* $^{-/-}$ mice were impaired in their response to LPS, with a 5-fold reduction in the percentage of cells that produced IFN- γ , measured by ICCS (Fig. 2A). *IL-12p40* $^{-/-}$ mice are deficient for the p40 subunit of IL-12, which is shared by IL-23, but the earlier study using blocking IL-12 Abs (16) would suggest that it is IL-12 that is required.

Because the absence of IL-12p40 did not completely eliminate IFN- γ production by $V\alpha 14i$ NKT cells after LPS injection, we examined the effect of IL-18 deficiency, since earlier studies have shown that $V\alpha 14i$ NKT cells produce cytokines when exposed to

IL-18 in vitro (21). Also, IL-18 synthesis is strongly induced by LPS, and the combination of IL-12 and IL-18 is known to have potent stimulatory effects on IFN- γ production (22, 23). We therefore measured IFN- γ production by V α 14i NKT cells in response to LPS in *IL-18*^{-/-} mice (Fig. 2B). V α 14i NKT cells from *IL-18*^{-/-} mice had an even more pronounced impairment in IFN- γ production after LPS (Fig. 2B). No enhancement in IL-4 production in response to LPS by V α 14i NKT cells from *IL-12p40*^{-/-} and *IL-18*^{-/-} mice was observed (data not shown). This defect in IFN- γ production by V α 14i NKT cells in *IL-12p40*^{-/-} and *IL-18*^{-/-} mice was not due to an intrinsic defect in the capacity of V α 14i NKT cells to produce cytokines, as they responded normally to α GalCer injection (data not shown).

Because type I IFNs are involved in the production of IFN- γ by memory CD8⁺ T cells in response to LPS (24), the role of type I IFNs in the V α 14i NKT cell response to LPS was tested using mice that are unresponsive to type I IFNs (*IFNAR1*^{-/-}). Percentages of IFN- γ -producing V α 14i NKT cells observed by ICCS 6 h after injection in *IFNAR1*^{-/-} mice were slightly reduced but still comparable to wild-type mice (data not shown), suggesting that type I IFN responsiveness was not required for V α 14i NKT cells to respond to LPS. These findings indicate that IFN- γ production by V α 14i NKT cells in response to LPS requires APC activation and the consequent production of cytokines such as IL-12 or IL-23 and IL-18.

CD1d Ag presentation is not required to induce V α 14i NKT cell IFN- γ

We tested whether CD1d recognition in addition to cytokines was essential for the activation of mouse V α 14i NKT cells by LPS. To do this, we cultured purified V α 14i NKT cells, obtained by positive selection, with BM DCs in the presence or absence of LPS. Cytokine production was measured in the supernatants after 48 h (Fig. 3, A and B). In one protocol, V α 14i NKT cells were sorted from mouse spleens using α GalCer-CD1d tetramers and Abs to TCR β . Neither BM DCs nor V α 14i NKT cells cultured alone with LPS produced IFN- γ , nor did V α 14i NKT cells cultured together with BM DCs without LPS (data not shown). IFN- γ was produced when V α 14i NKT cell-DC cocultures contained LPS (Fig. 3A). Surprisingly, addition of the anti-CD1d blocking Ab 1B1 had only a slight effect on IFN- γ production in this culture, and DCs from *CD1d*^{-/-} mice were almost as effective at stimulating IFN- γ in the presence of LPS as wild-type DCs (Fig. 3A). However, DCs from *IL-12p40*^{-/-} mice were poor activators in the V α 14i NKT cell cocultures with LPS (Fig. 3A), consistent with the reduced cytokine production by V α 14i NKT cell observed in the ex vivo analysis of cells from *IL-12p40*^{-/-} mice (Fig. 2A).

V α 14i NKT cells sorted with α GalCer-CD1d tetramers are partially activated, as judged by a modest amount of IL-4 secretion (ranging from 100 to 200 pg/ml in five independent experiments, data not shown) when cultured in vivo without further stimulation. It is therefore possible that the enriched V α 14i NKT cells (>97% purity) received a TCR signal during their enrichment. We used two additional purification schemes to address this issue. In one set of experiments, NKT cells were positively selected from the spleen using Abs to NK1.1 and TCR β . Although this also could cause some degree of TCR engagement, unlike cells positively selected with the tetramer, these NKT cells did not produce IL-4 in vitro (data not shown). NKT cells purified in this way therefore were cultured with *CD1d*^{-/-} BM DCs and LPS (data not shown), and the absence of CD1d expression by the DC had little effect on IFN- γ production. Because V α 14i NKT cells express CD1d, although much lower amounts than BM DC, it is possible that V α 14i NKT cells present glycolipid self-Ag to one another. Therefore, we

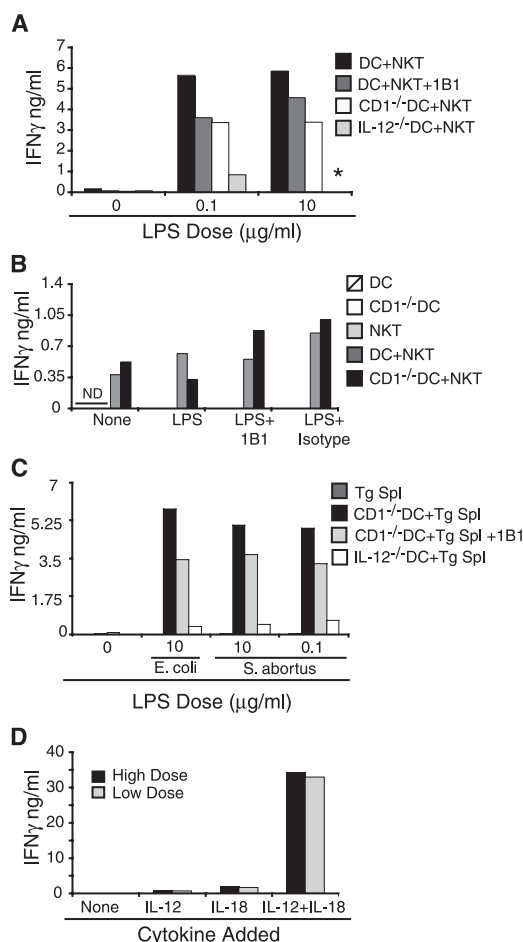


FIGURE 3. Ag presentation by CD1d is not required for IFN- γ production by V α 14i NKT cells in response to LPS. **A** and **B**, V α 14i NKT cells (NKT) were cultured with LPS and either wild-type (DC), *CD1d*^{-/-} (*CD1d*^{-/-} DC) or *IL-12p40*^{-/-} (*IL-12*^{-/-}) BM-derived DCs, in the presence or absence of anti-CD1d blocking Abs (1B1). V α 14i NKT cells were sorted based on staining with one of two schemes: α GalCer-CD1d tetramer (**A**) and anti-TCR β Abs or anti-NK1.1 and anti-CD5 Abs (**B**). The dose of LPS used in **B** is 10 μ g/ml. No IFN- γ production was detected in cultures that contained either BM-derived DCs or V α 14i NKT cells alone. **C**, V α 14i NKT cells were enriched from the spleens of V α 14 Tg mice (Tg Spl) and cultured with LPS from either *E. coli* or *S. abortus equi* (*S. abortus*) and BM-derived DCs from either *CD1d*^{-/-} (*CD1d*^{-/-} DC) or *IL-12p40*^{-/-} (*IL-12*^{-/-}). One representative of two experiments is shown. **D**, Sorted V α 14i NKT cells were cultured, without BM-derived DCs, with two different doses of rIL-12 and IL-18 (high dose: 10 ng/ml IL-12 and 100 ng/ml IL-18. Low dose: 100 pg/ml IL-12 and 1 ng/ml IL-18). IFN- γ levels after 48 h of culture were measured in the supernatants by ELISA. Each panel represents one of at least three independent experiments. *, Not done.

added anti-CD1d Ab to the LPS-containing cocultures in which *CD1d*^{-/-} BM DCs were used. Even under these conditions, when CD1d-mediated Ag presentation was most effectively blocked, similar levels of IFN- γ were elicited (data not shown).

In a second scheme, we used positive selection with Abs for NK1.1 and the inhibitory receptor CD5 (Fig. 3B). This procedure completely avoids any TCR engagement and the resulting population consists of 75% tetramer binding cells (data not shown). Coculture of this highly enriched population with *CD1d*^{-/-} BM DCs and LPS in the presence of anti-CD1d blocking Abs also led to IFN- γ secretion (Fig. 3B). To further ensure that V α 14i NKT cells were not activated during purification, these cells were enriched by negative selection from the spleens of mice transgenic

for the invariant V α 14 TCR (V α 14 Tg). B cells, CD8 T cells, macrophages, DCs, and $\gamma\delta$ T cells were depleted using lineage specific markers and NK cells were depleted by virtue of their CD11b expression (data not shown). The resulting population was ~15% V α 14i NKT cells (called Tg Spl), and was cultured with BM-derived DCs from *CD1d*^{-/-} and *IL-12p40*^{-/-} mice (Fig. 3C). Tg Spl cells produced IFN γ in response to LPS from either *E. coli* or *S. abortus equi*, even when cultured with *CD1d*^{-/-} BM derived DCs in the presence of blocking CD1d Abs (Fig. 3C), but the levels of IFN γ were strongly reduced when the BM derived DCs were from *IL-12p40*^{-/-} mice (Fig. 3C). These data indicate that CD1d-mediated Ag presentation is not required for IFN- γ production in cocultures of V α 14i NKT cells and BM DCs with LPS.

APC-derived cytokines are sufficient to drive V α 14i NKT cell activation

Because CD1d expression on DCs appeared to be unnecessary to induce IFN- γ in the DC-T cell cocultures containing LPS (Fig. 3, A–C), we tested whether cytokines alone were sufficient to activate V α 14i NKT cells. V α 14i NKT cells were sorted based on staining for the α GalCer-CD1d tetramer and TCR β , and the cells cultured with one of two concentrations of rIL-12 (100 pg/ml or 10 ng/ml), IL-18 (1 or 100 ng/ml), or with both cytokines (Fig. 3D), and production of IFN- γ by the cultured V α 14i NKT cells was measured 48 h later. The lower concentrations are physiologically relevant, because similar concentrations can be measured in the serum of mice after LPS injection (see Fig. 4 below). Addition of IL-12 or IL-18 individually led to the synthesis of measurable amounts of IFN- γ from V α 14i NKT cells, even at the lower dose of recombinant cytokines (Fig. 3D). Addition of both cytokines together resulted in strongly enhanced IFN- γ production (Fig. 3D), even at the lower doses of recombinant cytokines (Fig. 3D). V α 14i NKT cells cultured with rIL-12 or IL-18 or both cytokines in the presence of BM-derived DC showed comparable levels of IFN- γ production, indicating that any other LPS-independent APC-derived factors were not important (data not shown). NKT cells sorted based on expression of NK1.1 and TCR β were cultured with IL-12 and IL-18 with similar results (data not shown), excluding an activating role for the α GalCer-CD1d tetramer. In conclusion, the data are consistent with the hypothesis that the stimulation provided by DC for V α 14i NKT cells in vitro is entirely due to the IL-12 and IL-18 they produce, and that the two cytokines together, even in small quantities, are both necessary and sufficient for maximal IFN- γ production by V α 14i NKT cells.

Reduced innate immune response in mice lacking V α 14i NKT cells

Having characterized the V α 14i NKT cell response to LPS, we investigated how V α 14i NKT cell deficiency influenced the overall innate immune response to LPS. It has been reported that CD1d expression is necessary for the progression of the generalized Shwartzman reaction (11), an animal model of septic shock in which an initial priming dose (in this model, 40 μ g) of LPS is followed 24 h later by a higher (400 μ g) challenge dose, resulting in mortality. One other study, however, did not come to a similar conclusion (12). In agreement with the second study, we also found that *CD1d*^{-/-} mice on the C57BL/6 background did not have decreased mortality following LPS challenge, with 4 of 11 (36.6%) *CD1d*^{-/-} mice surviving compared with 4 of 15 (26.6%) wild-type control animals. In contrast, when *Ja18*^{-/-} mice on the C57BL/6 background were analyzed, they were protected, with 11 of 15 (76.6%) surviving. Kaplan-Meier survival curve analysis showed that the survival of *Ja18*^{-/-} mice was statistically significant when compared with survival of wild-type mice (p =

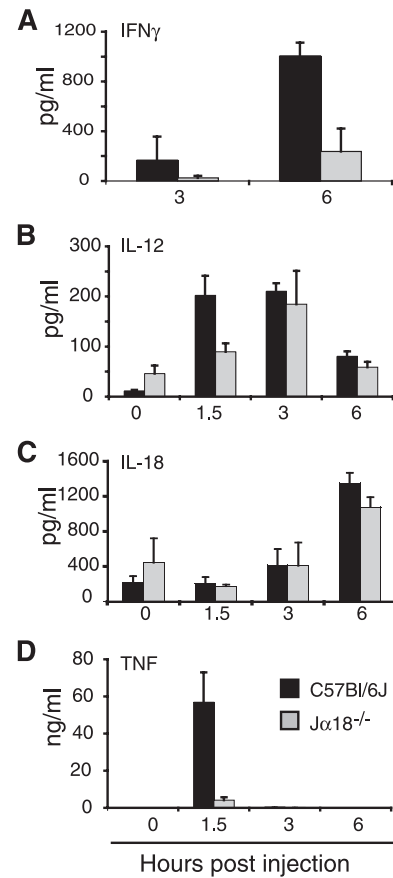


FIGURE 4. V α 14i NKT cell-deficient *Ja18*^{-/-} mice have defects in the production of proinflammatory cytokines in response to LPS. A–D, C57BL/6J or *Ja18*^{-/-} mice were injected with LPS and bled at the indicated time points. Cytokine levels in the serum were measured by ELISA (A and C) or using the Bioplex system (B and D), as described in detail in *Materials and Methods*. A, Data shown are averages from two mice, from one experiment of three. B–D, Data shown are the averages of measurements from three mice per group.

0.0120), but the survival of *CD1d*^{-/-} mice was not (p = 0.6038). A dichotomy in the results obtained comparing *Ja18*^{-/-} and *CD1d*^{-/-} mice has been interpreted in other experimental systems as reflecting a difference between the effects of lacking only V α 14i NKT cells (*Ja18*^{-/-} mice), and the additional effects of lacking other CD1d-restricted T cells with more diverse TCRs, sometimes called type II NKT cells, in the *CD1d*^{-/-} mice (25, 26). In several experimental systems, such as the recurrence of lymphoma growth (25), these type II NKT cells have been shown to have functional capabilities different from V α 14i NKT cells. Regardless, using survival as the endpoint clearly has variable results in different studies, perhaps because of differences in the LPS used, mouse genetic background, or conditions in different vivaria, and does not provide much quantitative insight into the in vivo response to LPS. We therefore focused our analysis on the response to the priming dose of LPS in the *Ja18*^{-/-} strain and control mice, because this strain provides the most precise ablation of V α 14i NKT cells.

Ja18^{-/-} and wild-type control mice were injected with LPS and serum cytokines were measured at the indicated time points after injection, by either cytometric bead array (Fig. 4, B and D) or by ELISA (Fig. 4, A and C). The amounts of serum IL-12p70 (Fig. 4B) and IL-18 (Fig. 4C) in *Ja18*^{-/-} mice were comparable to those in wild-type mice at the early hours following LPS injection, while IFN- γ levels 6 h after LPS (Fig. 4A) were strongly reduced

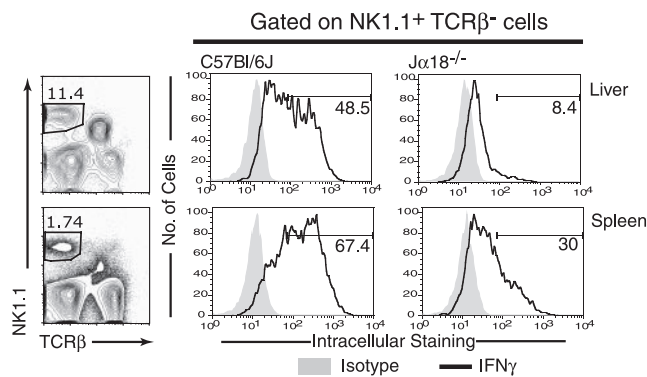


FIGURE 5. NK cells from $J\alpha 18^{-/-}$ mice display impaired IFN- γ production in response to LPS. C57BL/6J or $J\alpha 18^{-/-}$ mice were injected with LPS and spleens and livers harvested after 6 h. IFN- γ production was detected by ICCS performed directly ex vivo without any further treatments, and compared with intracellular staining with the appropriate isotype control Ab. The numbers indicate percentages of intracellular IFN- γ^{+} cells. The gates for intracellular IFN- γ levels are set based on intracellular IFN- γ staining in NK cells from control PBS-injected mice (data not shown). Panels on the left show the NK cell gate. Data shown are from one representative mouse, at least five mice were tested in each case.

in $J\alpha 18^{-/-}$ mice. The most striking difference, however, was in the amount of serum TNF (Fig. 4D), with a nearly 10-fold reduction in $J\alpha 18^{-/-}$ mice at 1.5 h after LPS. A similar reduction in serum TNF levels in $J\alpha 18^{-/-}$ mice was observed in additional experiments, where cytokine levels were measured by ELISA rather than cytometric bead array (data not shown). Because IL-12p70 and IL-18 levels are similar in wild-type and $J\alpha 18^{-/-}$ mice, the impaired production of these cytokines cannot explain the reduction in serum IFN- γ and TNF seen in $J\alpha 18^{-/-}$ mice, and the data are consistent with the hypothesis that $V\alpha 14i$ NKT cells act downstream of TLR4-mediated activation of APC, and respond to the IL-12 and IL-18 produced by those APC to stimulate IFN- γ and TNF synthesis.

Impaired LPS activation of innate immune cells in $J\alpha 18^{-/-}$ mice

We considered it unlikely that $V\alpha 14i$ NKT cells were directly responsible for the high levels of IFN- γ and TNF in the serum of mice injected with LPS. NK cells have been shown to be the principal source of serum IFN- γ after LPS (27), and by ICCS, $V\alpha 14i$ NKT cells did not produce TNF in response to LPS (data not shown). This suggests that activated $V\alpha 14i$ NKT cells rapidly induce cytokine production by other cell types, by analogy to their rapid activation of NK cells after α GalCer injection (28–30).

IFN- γ production by NK cells from wild-type and $J\alpha 18^{-/-}$ mice was examined by ICCS 6 h after LPS injection (Fig. 5). As was done for the analysis of $V\alpha 14i$ NKT cells, the gated (NK1.1 $^{+}$, TCR β^{-}) NK cells were analyzed immediately ex vivo without further stimulation or the use of brefeldin A. The majority of NK cells, expressed intracellular IFN- γ 6 h after LPS, but this percentage was strongly reduced in $J\alpha 18^{-/-}$ mice, particularly in the liver (Fig. 5). By ICCS analysis, however, NK cells were not major producers of TNF (data not shown). Previous studies have identified neutrophils and macrophages as some of the primary sources of TNF produced in response to LPS (31), therefore these cell types were tested for TNF production after LPS in $J\alpha 18^{-/-}$ mice. Because of the very early appearance of TNF in the serum, ICCS analysis for TNF synthesis was conducted 45 min after LPS injection (Table I). Although the data varied between experiments, the percentage of splenic neutrophils (CD11b high Gr-1 high) and macro-

Table I. Percentages of intracellular TNF $^{+}$ cells 45 min after LPS injection

Expt. No.	Neutrophils		Macrophages	
	C57BL/6J	$J\alpha 18^{-/-}$	C57BL/6J	$J\alpha 18^{-/-}$
1	18.7 ^a	5.91	13.8	1.93
2	40.8	26.4	13.1	9.89
3	26.2	13.8	14.4	8.21

^a The numbers indicate the percentages of TNF $^{+}$ cells among the indicated cell populations in the spleen. Neutrophils were gated as CD11b high Gr-1 high and macrophages as CD11b low Gr-1 low F4/80 $^{+}$. Each experiment had one mouse per group. Three independent experiments are shown here.

phages (CD11b low Gr-1 low F4/80 $^{+}$, also verified to be CD68 high) from $J\alpha 18^{-/-}$ mice that produced TNF was consistently lower than that seen in wild-type mice (Table I), suggesting that a defect in myeloid cell-derived TNF was the cause of the reduction seen in systemic TNF in $J\alpha 18^{-/-}$ mice.

IFN- γ augments the proinflammatory cascade

We hypothesized that IFN- γ production by $V\alpha 14i$ NKT cells was in part responsible for the augmentation of the innate immune response to LPS. IFN- γ is known to be important for the induction of TNF in the inflammatory response (32–34) and IFN- $\gamma^{-/-}$ mice had a strong reduction in serum TNF 1.5 h after LPS injection (data not shown). Moreover, in the context of glycolipid Ag stimulation of their TCR, the immediate production of IFN- γ by $V\alpha 14i$ NKT cells contributes to the rapid activation of NK cells and other cell types (28–30, 35). Although expression of activation markers such as CD69 by $V\alpha 14i$ NKT cells was increased at early time points after LPS injection, at time points as early as 1 h, the cells were negative for intracellular IFN- γ when analyzed directly ex vivo (data not shown). Because TNF production by myeloid cells is already robust by 1 h, it therefore appeared that the bulk of the IFN- γ synthesis by $V\alpha 14i$ NKT cells occurs too late to be responsible for the early wave of myeloid cell TNF production. As noted above, our ICCS protocol should closely reflect the in vivo situation, but it is not a highly sensitive assay, and therefore it may not be able to detect lower amounts of early cytokine synthesis. To determine whether $V\alpha 14i$ NKT cells express detectable IFN- γ at very early time points after LPS injection, these cells were sorted from the livers and spleens of mice 1 h after LPS injection, and IFN- γ mRNA expression was measured by real-time PCR (Fig. 6A). IFN- γ mRNA was enhanced in $V\alpha 14i$ NKT cells 1 h after LPS injection compared with similarly prepared cells from control mice, with a 5-fold level of enhancement seen in the liver (Fig. 6A).

To test whether reduced early IFN- γ synthesis was responsible for the reduction in serum TNF seen in response to LPS in $J\alpha 18^{-/-}$ mice, these mice were injected with rIFN- γ together with LPS, and TNF production in the serum was measured after 1.5 h (Fig. 6B). A total of 100 ng/mouse of rIFN- γ was sufficient to induce an ~ 2 -fold increase in serum TNF when coadministered with LPS (Fig. 6B). These observations suggest that the reduction in serum TNF observed in $J\alpha 18^{-/-}$ mice in response to LPS may be due to reduced early production of IFN- γ , and taken together, they further suggest that the early synthesis of IFN- γ by $V\alpha 14i$ NKT cells could be a critical factor for the full activation of the proinflammatory cascade in response to LPS.

Discussion

There is abundant evidence that $V\alpha 14i$ NKT cells are activated by microbial products such as LPS (4, 15, 16), but here we provide a

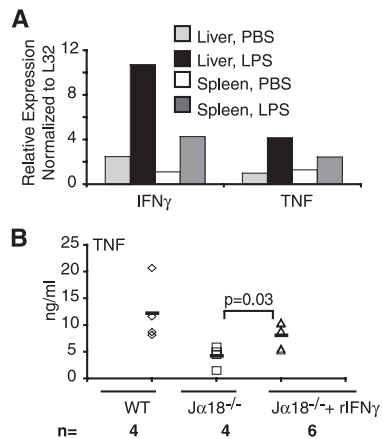


FIGURE 6. IFN- γ from V α 14i NKT cells may augment the production of TNF in response to LPS. **A**, RNA was purified from V α 14i NKT cells sorted from the spleens or livers of C57BL/6J mice that had been injected 1 h earlier with either PBS or LPS. IFN- γ or TNF message levels were measured by real-time PCR. Data shown are from one of two independent experiments. **B**, C57BL/6J or Ja18^{-/-} mice were injected with LPS, and another cohort of Ja18^{-/-} mice was injected with LPS together with 100 ng of recombinant IFN- γ (Ja18^{-/-} + rIFN γ). The mice were bled 1.5 h after injection and TNF levels in the serum measured by ELISA. The numbers of mice per group are indicated beneath the x-axis. Data shown are from one of two independent experiments.

novel mechanism for this response. We showed that IL-12 and IL-18 from activated APC are necessary and sufficient for the rapid activation of V α 14i NKT cells. Additionally, we showed how the cytokine-driven stimulation of V α 14i NKT cells to produce IFN- γ , and perhaps other products, led to the activation within hours of other innate immune cells. V α 14i NKT cells thereby amplify the innate immune response to LPS, with potentially important consequences for the induction of septic shock. The cytokine-dependent activation of V α 14i NKT cells reported here is consistent with several previous studies that were mostly conducted in vitro (21), but these investigations did not rule out the concomitant recognition of a self-Ag presented by APC or by the V α 14i NKT cells themselves.

A striking observation is that CD1d-mediated Ag presentation was not required to activate V α 14i NKT cells in this system. This observation runs counter to published results (4, 16) that demonstrated the need for CD1d to present an endogenous Ag to induce activation of V α 14i NKT cells. Two different mechanisms have been proposed previously for the activation of V α 14i NKT cells by microbial products that do not contain a ligand for the invariant TCR. First, it has been shown that V α 14i NKT cells can be stimulated by the combined action of IL-12 from bacteria-activated APC and endogenous GSL Ag(s) presented by CD1d (16). The requirement for self-Ag recognition was inferred from the ability of anti-CD1d mAbs to block the V α 14i NKT cell response to *Salmonella typhimurium* (16). Furthermore, BM DCs from *Hexb*^{-/-} mice, which cannot generate the autologous Ag isoglobotrihexosyl ceramide (36), could not stimulate V α 14i NKT cell cytokine production in response to *Salmonella* (4). Second, self-Ag presentation could be sufficient to activate V α 14i NKT cells, as illustrated by the response to *Schistosoma* eggs, which required APC to express the *Hexb* gene product but not IL-12 (37), although in this system other forms of innate immune activation that were not MyD88 dependent could have been going on simultaneously.

The TCR-independent mode of bacterial recognition described here provides a third mechanism, not mutually exclusive with

those described above. Overall, the data indicate that V α 14i NKT cells indirectly sense the presence of microbes in several ways, in that they can apparently be activated by the combination of APC-derived cytokines and self-Ag presented by CD1d, or by either factor alone. We hypothesize that the mechanism by which V α 14i NKT cells are activated during infection depends critically on the nature and strength of the microbial stimulus and the APC used, and the extent to which this stimulus activates IL-12 and IL-18, or self-Ag presentation. Lysates of whole organisms or *Schistosoma* eggs have the capacity to activate many pathways, not just those downstream of TLR4, and the APCs in those studies therefore were likely to be activated differently. Recent studies have shown that a combination of signals can serve to increase CD1d expression by macrophages (38), and the same might be true of DC. It is possible, therefore, that some products from microbes may optimally stimulate CD1d expression and endogenous GSL presentation, and that under those circumstances, IL-12 and IL-18 may not be required for V α 14i NKT cell activation.

In yet another mechanism it has been proposed that V α 14i NKT cells express TLR4 and that they can be activated by LPS to produce IL-4 within minutes of stimulation (15). We could not find evidence for either TLR4 or CD14 expression by V α 14i NKT cells. Although it is possible that a very low level of expression could have escaped detection, we do not believe it to be sufficient to stimulate V α 14i NKT cell IFN- γ production as purified V α 14i NKT cells cultured with LPS did not produce detectable amounts of cytokines (data not shown). In agreement with a previous study (16), we found that V α 14i NKT cell activation to produce IFN- γ is not a direct effect of LPS on V α 14i NKT cells, but it requires prior APC activation.

An intriguing finding to emerge from these studies is the extent to which the presence of V α 14i NKT cells is needed to fully activate other cell types within hours during the response to LPS. Considering the relatively small number of V α 14i NKT cells, and the evidence that LPS does not activate them directly, the degree to which they regulate the activation of other innate cells, and the rapidity of this response amplification, are astonishing. The mechanism has not been completely determined, but we have evidence indicating that V α 14i NKT cells produce a low level of IFN- γ within an hour of LPS injection, and furthermore, exogenous IFN- γ restores TNF production in response to LPS in Ja18^{-/-} mice. We suggest the existence of a positive feedback loop involving V α 14i NKT cells and innate immune cells where LPS activates APCs that express TLR4 and rapidly induces the production of IL-12 and IL-18. This IL-12 and IL-18 then activate early IFN- γ production from V α 14i NKT cells. This IFN- γ from V α 14i NKT cells feeds back to the APCs to potentiate TNF production, and induce further APC activation. V α 14i NKT cell-derived IFN- γ is also apparently necessary to fully activate NK cells to produce IFN- γ , similar to the effect of V α 14i NKT cell-derived IFN- γ on NK cell activation in response to α GalCer (30).

The increased systemic production of TNF and IFN- γ in mice that have normal numbers of V α 14i NKT cells has important consequences in vivo, as Ja18^{-/-} mice deficient for V α 14i NKT cells were resistant to the generalized Shwartzman reaction in our experiments and in those conducted by several other groups. Also, recent work by another group demonstrated that activation of V α 14i NKT cells alone by α GalCer injection can substitute for the priming dose of LPS in the Shwartzman reaction (39). These results taken together suggest that V α 14i NKT cells play an active role during the response to LPS, rather than a passive role in conditioning innate immune cells to ensure optimal responsiveness to LPS.

In conclusion, we have demonstrated a third pathway by which $V\alpha 14i$ NKT cells indirectly sense the presence of microbial products. Even in the absence of a TCR-mediated signal, these T lymphocytes can act in concert with innate immune cells to amplify the innate immune response, while in other cases, TCR-mediated recognition of self-Ag may be necessary or sufficient. The flexible ability of $V\alpha 14i$ NKT cells to be activated rapidly in different ways by microbial products ensures that these cells will be activated by diverse microbes, marking them as crucial components of the innate immune response to infection.

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Disclosures

The authors have no financial conflict of interest.

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