Activation of decidual invariant natural killer T cells promotes lipopolysaccharide-induced preterm birth

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ABSTRACT: Invariant natural killer T (iNKT) cells are crucial for host defense against a variety of microbial pathogens, but the underlying mechanisms of iNKT cells activation by microbes are not fully explained. In this study, we investigated the molecular mechanisms of iNKT cell activation in lipopolysaccharide (LPS)-stimulated preterm birth using an adoptive transfer system and diverse neutralizing antibodies (Abs) and inhibitors. We found that adoptive transfer of decidual iNKT cells to LPS-stimulated iNKT cell deficient Jα18−/− mice that lack invariant Vα14Ja281T cell receptor (TCR) expression significantly decreased the time to delivery and increased the percentage of decidual iNKT cells. Neutralizing Abs against Toll-like receptor 4 (TLR-4), CD1d, interleukin (IL)-12 and IL-18, and inhibitors blocking the activation of nuclear factor κB (NF-κB), mitogen-activated protein kinase (MAPK) p38 and extracellular signal-regulated kinase (ERK) significantly reduced in vivo percentages of decidual iNKT cells, their intracellular interferon (IFN)-γ production and surface CD69 expression. In vitro, in the presence of the same Abs and inhibitors used as in vivo, decidual iNKT cells co-cultured with LPS-pulsed dendritic cells (DCs) showed significantly decreased extracellular and intracellular IFN-γ secretion and surface CD69 expression. Our data demonstrate that the activation of decidual iNKT cells plays an important role in inflammation-induced preterm birth. Activation of decidual iNKT cells also requires TLR4-mediated NF-κB, MAPK p38 and ERK pathways, the proinflammatory cytokines IL-12 and IL-18, and endogenous glycolipid antigens presented by CD1d.

Key words: natural killer T cells / activation / preterm birth / lipopolysaccharide / inflammation

Introduction

The incidence of preterm birth in developed countries is 5% and may be as high as 11.1% in developing countries (Makieva et al., 2014). Such deliveries are a major cause of morbidity and mortality in affected mothers and babies. Inflammation is considered a significant contributor to preterm birth (Bastek et al., 2011).

Natural killer T (NKT) cells are a subset of innate-like T lymphocytes that influence a broad spectrum of immunological responses (Van Kaer et al., 2013). NKT cells are a T lymphocyte subset that is characterized by expression of an invariant T cell receptor (TCR) α-chain, formed by a Vα14 to Ja18 rearrangement in mice (Pellicci et al., 2009). When paired with several β-chains, predominantly Vβ8.2, this α-chain imparts specificity for glycolipids presented by CD1d, a major histocompatibility complex (MHC) class I-like antigen-presenting molecule (Kronenberg and Kinjo, 2009; Van Kaer et al., 2013). These cells are commonly referred to as type I or invariant NKT (iNKT) cells. Engagement of the TCR on iNKT cells leads to rapid and robust cytokine secretion, including the characteristic Th1 and Th2 cytokines, interferon (IFN)-γ and interleukin (IL)-4, respectively (Wu and Van Kaer, 2011).

iNKT cell-derived cytokines activate many other immune cell types, including dendritic cells (DCs), macrophages, natural killer (NK) cells, conventional CD4+ T cells, CD8+ T cells, B cells and neutrophils (Brennan et al., 2013). iNKT cells participate in a variety of immune responses, such as microbial immunity, tumor immunity, autoimmunity and allergic diseases (Brennan et al., 2013).

iNKT cells are crucial for host defense against a variety of microbial pathogens. How CD1d-restricted iNKT cells, with their limited TCR repertoire, become activated in response to vastly diverse microbial infections is not completely understood. Two distinct pathways have been proposed for iNKT cell activation. The TCR-mediated pathway involves direct recognition of CD1d-presented microbial glycolipid antigens, such as glycosphingolipids from Sphingomonas (Kinjo et al. 2005; Mattner et al., 2005) and diacylglycerols from Borrelia burgdorferi, Ehrlichia and Streptococcus pneumoniae (Kinjo et al., 2006, 2011). In contrast to the direct recognition of microbial glycolipids, iNKT cells can be fully...
activated by proinflammatory cytokines, such as IL-12, IL-18 or type I IFNs secreted by antigen-presenting cells upon stimulation with Toll-like receptor (TLR) ligands, with (Brigli et al., 2003; Mattner et al., 2005; Paget et al., 2007; Salio et al., 2007) or without (Nagarajan and Kronenberg, 2007; Tyni, 2008; Wesley et al., 2008) a weak TCR-mediated signal provided by recognition of CD1d-presented self-antigens.

TLRs are type I transmembrane receptors that respond to a wide range of microbial components, and are expressed by sentinel cells of the immune system such as DCs and macrophages (Buxadé et al., 2012). Upon ligand stimulation, TLRs trigger inflammatory signaling that usually begins with the recruitment of an adaptor protein called myeloid differentiating factor 88 (MyD88) (Bonham et al., 2014). Activation of MyD88 induces downstream signaling cascades involving the phosphorylation of mitogen-activated protein kinase (MAPK) p38, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), the activation of nuclear factor κB (NF-κB), and finally the release of proinflammatory cytokines (Tamura et al., 2011; O’Neill et al., 2013).

Conventional T cells do not express the invariant Vα14-Jα18 antigen receptor (Tanguchi et al., 1996), indicating their selective usage in Vα14 NKT cells. Thus, disruption of the invariant Vα14-Jα18 receptor results in the selective loss of Vα14 NKT cells, leaving other types of lymphocytes, including T, B, and NK cells, intact (Ito et al., 2000). Other than a complete loss of the Vα14 NKT cell population, Jα18 knockout (KO) mice, which are fertile and healthy in appearance (Rogers et al., 2008), exhibit no alterations in total lymphocyte populations when compared with wild type (WT) mice (Ito et al., 2010; Ito et al., 2012). Our previous studies using a lipopolysaccharide (LPS)-injected inflammatory preterm birth model and Jα18 KO mice have demonstrated that iNKT cells play a role in inflammation-associated preterm birth by enhancing the expression of co-stimulatory molecules on DCs and by activating DCs, T cells and NK cells at the maternal–fetal interface (Li et al., 2012). In the present study, we employed decidual iNKT cell adoptive transfer and canonical neutralizing antibodies (Abs) and inhibitors that block key response pathways, which range from immune recognition to effector cytokine production, to further elucidate the roles of decidual iNKT cells and the molecular mechanisms of decidual iNKT activation in inflammation-induced preterm birth. Our data demonstrate that the activation of decidual iNKT cells is dependent on TLR4-mediated NF-κB, p38, ERK, IL-12 and IL-18 pathways, in combination with autoantigenic TCR stimulation and plays an important role in inflammation-induced preterm birth.

Materials and Methods

Reagents

Phycoerythrin (PE)-conjugated α-galactosylceramide (α-GalCer)-loaded CD1d tetramer, anti-PE microbeads, B220 microbeads, dead cell removal kit, FcR blocking reagent and pan dendritic cell isolation kit were purchased from BioLegend (San Diego, CA, USA). Anti-mouse IL-12 Ab and rat IgG2a κ isotype control Ab were purchased from eBioscience (San Diego, CA, USA). Anti-mouse IL-18 Ab, rat IgG1 isotype control Ab and IL-18 enzyme-linked immunosorbent assay (ELISA) kit were purchased from MBL International (Nagoya, Japan). Recombinant mouse IL-12 and IL-18 were purchased from R&D Systems (Minneapolis, MN, USA). IL-12p70, IFN-γ and IL-4 ELISA kits were purchased from BD Biosciences (San Jose, CA, USA). Dispase II was purchased from Roche (Basel, Switzerland). Ficoll-Paque Premium was purchased from GE Healthcare (Pittsburgh, PA, USA).

Animals

Eight-week-old female and male C57BL/6 mice were purchased from the Experimental Animal Center of Zhongshan University (Guangzhou, China). iNKT cell-deficient Jα18 KO mice on a C57BL/6 background were purchased from CnsGen Biotech (Beijing, China). Mice were bred in the Laboratory Animal Center of Guangzhou Medical University (Guangzhou, China) in pathogen-free conditions. Syngeneic C57BL/6 x C57BL/6 and Jα18 KO x Jα18 KO mating combinations were established. Each female mouse was co-caged with one male. The point at which a vaginal plug was detected was designated as Day 0 of gestation.

Ethical approval

All animal protocols were approved by the Animal Care and Use Committee of Guangzhou Medical University.

Generation of decidual iNKT cells

Mouse decidual iNKT cells were isolated from murine C57BL/6 decidual mononuclear cell (MNCs) using a PE-conjugated α-GalCer-loaded CD1d tetramer and anti-PE microbeads after depletion of B cells using B220 microbeads according to the manufacturer’s instructions (Miltenyi Biotec). Briefly, decidual MNC suspensions were prepared and dead cells were removed using a dead cell removal kit. The single cell suspensions were then labeled with B220 microbeads and B cells were depleted using an LD column and an autoMACS™ separator (Miltenyi Biotec). The unlabeled B cell-depleted fractions were collected. An FcR blocking reagent was added to avoid Fc receptor-mediated Ab labeling. The B cell-free fractions were then labeled with PE-conjugated α-GalCer-loaded CD1d tetramer, washed and enriched for CD1d-α-GalCer tetramer+ iNKT cells using anti-PE-microbeads, two MS columns and an autoMACS™ separator. The percentage of iNKT cells in the enriched fraction was determined by labeling with APC/Cy7-conjugated CD3 and PE-conjugated α-GalCer-loaded CD1d tetramer. The purity of sorted iNKT cells was defined as the percentage of CD1d-α-GalCer tetramer+ CD3+ cells in the CD3+ cell population and routinely exceeded 97%, as determined using flow cytometry.

The LPS-induced preterm birth model and adoptive transfer of iNKT cells

As described previously (Li et al., 2012), on Day 15 of gestation, pregnant C57BL/6 mice or Jα18 KO mice were injected i.p. with 200 μL LPS in a saline solution (100 μg/kg body weight), taking care not to enter the amniotic cavity. We assessed correct i.p. injection in two ways. First, we aspirated the needle point was within the abdominal space. Next, we inspected the injection after withdrawal to detect blood that would indicate possible organ damage. Pregnant WT mice and Jα18 KO mice injected i.p. with 200 μL
PBS served as negative controls. For iNKT cell reconstitution of Jα18 KO mice, 2 × 10⁵ purified decidual iNKT cells from C57BL/6 mice were injected i.v. into the tail vein of Jα18 KO mice 2 h prior to LPS or PBS injection. The time to delivery was recorded and defined as the number of hours from the time of injection to delivery of the first pup. In addition, delivered LPS-treated Jα18 KO mice with or without iNKT cell adoptive transfer and PBS-treated Jα18 KO mice with iNKT cell adoptive transfer were sacrificed, and the percentages of decidual iNKT cells in CD3⁺ T cells were analyzed using flow cytometry.

In vivo treatment with neutralizing Abs and inhibitors

WT mice were injected i.p. with a variety of neutralizing Abs and inhibitors on Day 11 and Day 13 of gestation, and then injected with LPS on Day 15 of gestation. These neutralizing monoclonal Abs and inhibitors included: 6 μg anti-mouse TLR4 Ab, 50 μg anti-mouse CD1d Ab, 150 μg anti-mouse IL-12 Ab, 50 μg anti-mouse IL-18 Ab, 50 mg/kg PDTC, 25 mg/kg SB203580, 10 mg/kg PD98059 and 20 mg/kg SP600125. Mice that were injected with DMSO or isotype control Abs on Days 11 and 13 of gestation, and PBS on Day 15 of gestation served as negative controls. Mice that were treated with DMSO or isotype control Abs on Day 11 and 13 of gestation, and LPS on Day 15 of gestation served as positive controls. Delivered mice were sacrificed when delivered pups were found in the cage. Decidual MNCs were isolated as described below. The percentages of iNKT cells among CD3⁺ T cells were measured using flow cytometry.

For intracellular cytokine analysis, isolated decidual MNCs (10⁶ cells/ml) were incubated with 50 ng/ml PMA and 500 ng/ml ionomycin at 37°C in 5% CO₂ for 6 h. For the last 4 h of incubation, brefeldin A was added at a final concentration of 5 μg/ml to allow for intracellular accumulation of cytokines. iNKT cell intracellular cytokine production was analyzed using flow cytometry.

Isolation of decidual MNCs

The uterine horns of pregnant mice were opened longitudinally, and the entire placental and decidual units were separated individually from the corresponding embryo and its implantation site. After washing in PBS, pooled decidual tissues were cut into small pieces and digested three times with 1 mg/ml Dispase II at 37°C for 20 min each cycle in a shaking water bath. When single or clumps of cells were observed under the microscope, the released cells were separated from undigested tissue pieces by filtering through a 40-μm-pore nylon mesh strainer (Thermo Fisher Scientific, Waltham, MA, USA). MNCs were purified over a Ficoll-Paque Premium by centrifugation at 400 × g for 40 min at 20°C.

![Figure 1](image-url) Effects of iNKT cell adoptive transfer on the time to delivery and the percentages of decidual iNKT cells. Pregnant WT or Jα18 KO mice were injected i.p. with LPS or PBS on Day 15 of gestation. For iNKT cell reconstitution of Jα18 KO mice, 2 × 10⁵ purified decidual iNKT cells from C57BL/6 mice were injected i.v. into the tail vein of Jα18 KO mice 2 h prior to LPS injection. (A) The time to delivery was determined. Data are presented as mean ± SD (n = 8). (B) A graphical summary of decidual iNKT cell percentages in CD3⁺ T cells is presented. Data are presented as mean ± SD of six independent experiments. (C) One representative experiment out of six is shown. Numbers indicate the percentages of CD1dα-GaCer tetramer⁺ CD3⁺ T cells in CD3⁺ T cells (%). *P < 0.01.
Isolation of decidual DCs and cell co-culture

Decidual DCs were isolated using a pan dendritic cell isolation kit according to the manufacturer’s instructions (Miltenyi Biotec). Briefly, decidual MNC suspensions from C57BL/6 mice were prepared. The single cell suspensions were then labeled with a pan dendritic cell biotin-Ab cocktail, washed and incubated with anti-biotin microbeads. The DCs were then magnetically separated using the autoMACS separator. The purity of sorted DCs routinely exceeded 98%. Isolated decidual DCs (2 × 10^5 cells/ml) were incubated with a panel of neutralizing Abs and inhibitors, including 0.5 μg/ml anti-mouse TLR4 Ab, 10 μg/ml anti-mouse CD1d Ab, 10 μg/ml anti-mouse IL-12 Ab, 10 μg/ml anti-mouse IL-18, 50 μM PDTC, 10 μM SB203580, 50 μM PD98059 and 20 μM SP600125 for 2 h, then stimulated with LPS (10 ng/ml) for 12 h and extensively washed. Decidual DCs treated with DMSO or isotype control Abs plus PBS served as negative controls. Decidual DCs treated with DMSO or isotype control Abs plus LPS served as positive controls. A total of 2 × 10^4 decidual DCs were co-cultured with 2 × 10^4 sorted decidual iNKT cells in a round-bottom 96-well plate at 37°C in 5% CO_2 for 24 h. The culture supernatants were collected, and levels of IFN-γ and IL-4 were measured using ELISA. Decidual iNKT cell intracellular IFN-γ and IL-4 secretion and surface CD69 expression were detected using flow cytometry. In addition, decidual DCs (2 × 10^5 cells/ml) were cultured without decidual iNKT cells for 24 h. The culture supernatants were collected, and levels of IL-12p70, IL-18, IFN-γ and IL-4 were measured using ELISA.

In selected experiments, purified decidual iNKT cells (2 × 10^5) were co-cultured with purified decidual DCs (2 × 10^4) pretreated with anti-CD1d (10 μg/ml) or mouse IgG1 isotype control Ab (10 μg/ml) in the presence of various doses of IL-12 or IL-18, 1 ng/ml IL-12 and serially diluted IL-18, or 1 ng/ml IL-18 and serially diluted IL-12. Twenty-four hours later, supernatants were analyzed for IFN-γ by ELISA.

Flow cytometry

Aliquots of 10^6 cells in 50 μl PBS were incubated with APC/Cy7-conjugated CD3 (0.25 μg), PE-conjugated α-GalCer-loaded CD1d tetramer (1 μg) and

![Figure 2](image_url)

**Figure 2** Comparison of decidual iNKT cell percentages and CD69 expression following in vivo LPS stimulation. C57BL/6 mice were injected i.p. with a panel of neutralizing Abs (or isotype control Abs) and inhibitors (or vehicle) in the presence or absence of LPS alone on Day 15 of gestation. Delivered mice were sacrificed and decidual MNCs were isolated. The percentages of decidual iNKT cells in CD3^+^ cells (A and B) and CD69 expression on decidual iNKT cells (C and D) were measured using flow cytometry. Data are presented as mean ± SD of six independent experiments. *P < 0.01; #P < 0.01 versus all the groups, except the SP600125 group; ΔP < 0.01 versus all the groups, except the DMSO + LPS group.
PE/Cy7-conjugated CD69 (1 μg) for 30 min at 4°C. After washing twice with PBS, cells were fixed in a fixation buffer. For intracellular cytokine staining, cells were resuspended in a permeabilization wash buffer and incubated with FITC-conjugated IFN-γ (1 μg) and APC-conjugated IL-4 (1 μg). Isotype controls were established using matched fluorescence-labeled isotype control Abs to avoid nonspecific staining. Immunostained cells were analyzed on a FACSCanter flow cytometer (BD Biosciences) using FACSDiva software (BD Biosciences). The percentages of CD1d-α-GalCer tetramer-CD3+ cells in the CD3+ cell population and the expression of CD69 on CD1d-α-GalCer tetramer-CD3+ cells were measured. CD1d-α-GalCer tetramer-CD3+ cells were gated and screened for intracellular IFN-γ and IL-4 production.

ELISA
Culture supernatants were collected and stored at −80°C for batched cytokine examination. The levels of IL-12p70, IL-18, IFN-γ and IL-4 were assessed using commercially available ELISA kits. All assays were conducted according to the manufacturer’s instructions.

Statistical analysis
All statistical analyses were performed using SPSS 19.0 software (IBM, Armonk, NY, USA). For multiple group comparisons, data were analyzed using one-way ANOVA with Bonferroni post-test when the variances were homogeneous or with Tamhane’s T2 post-test when the variances were not homogeneous. Independent Student’s t-test was used for comparisons between two groups. Results were expressed as mean ± SD. A P-value of <0.05 was considered significant.

Results
Comparison of the time to delivery
The time to delivery in LPS-treated WT mice was 40.5 ± 5.2 h, which was significantly shorter than PBS-treated WT (112.5 ± 12.8 h, P < 0.01) or Jα18 KO mice (112.9 ± 12.5 h, P < 0.01). The time to delivery was significantly increased in LPS-injected Jα18 KO mice (64.9 ± 9.2 h,

Figure 3 Comparison of decidual iNKT cell intracellular IFN-γ levels following in vivo LPS exposure. C57BL/6 mice were injected i.p. with a panel of neutralizing Abs (or isotype control Abs) and inhibitors (or vehicle) in the presence or absence of LPS alone on Day 15 of gestation. Delivered mice were sacrificed and decidual MNCs were isolated. Decidual iNKT cell intracellular IFN-γ production was analyzed using flow cytometry. (A) The percentages of iNKT cells positive for IFN-γ in mice pretreated with neutralizing Abs are shown. Data are presented as mean ± SD of six independent experiments. (B) The percentages of iNKT cells positive for IFN-γ in mice pretreated with inhibitors are shown. One representative experiment out of six is shown. Gray shaded histogram: isotype control. Black line: inhibitor treated. Numbers indicate the percentages of IFN-γ+ cells among the iNKT cells (%). (C) A graphical summary of decidual iNKT cell intracellular IFN-γ production in mice pretreated with inhibitors is presented. Data are presented as mean ± SD of six independent experiments. #P < 0.01 versus all the groups, except the SP600125 group; ΔP < 0.01 versus all the groups, except the DMSO+LPS group.
$P < 0.01$) compared with LPS-injected WT mice. Adoptive transfer of decidual iNKT cells from C57BL/6 mice notably decreased the time to delivery in LPS-treated Jα18 KO mice (40.4 ± 4.9 h, $P < 0.01$) to a level that did not differ significantly from LPS-stimulated WT mice. In addition, decidual iNKT cell adoptive transfer did not increase the time to delivery in PBS-treated Jα18 KO mice (107.8 ± 12.2 h) (Fig. 1A).

**Effects of iNKT cell adoptive transfer on decidual iNKT cell percentages**

To investigate the mechanisms involved in decidual iNKT cell-mediated inflammatory preterm birth, percentages of decidual iNKT cells in LPS-treated Jα18 KO mice with or without iNKT cell adoptive transfer were compared. There were very few iNKT cells in LPS-treated Jα18 KO mice without iNKT cell adoptive transfer. However, the percentage of decidual iNKT cells was significantly elevated when LPS-treated Jα18 KO mice received an iNKT cell adoptive transfer ($P < 0.01$). In addition, there were few decidual iNKT cells in PBS-treated Jα18 KO mice with iNKT cell adoptive transfer (Fig. 1B and C).

**Comparison of decidual iNKT cell percentages and CD69 expression following in vivo LPS stimulation**

LPS injection significantly increased the percentage of decidual iNKT cells ($P < 0.01$) and the expression of CD69 ($P < 0.01$), an early activation marker, on decidual iNKT cells in control mice. In order to explore the underlying molecular mechanisms of these effects, we used a variety of canonical neutralizing Abs and inhibitors to block specific key steps in TLR4 signaling, from immune recognition and CD1d engagement to the generation of effector cytokines. Upon pretreatment with anti-TLR4 Ab, anti-CD1d Ab, anti-IL-12 Ab, anti-IL-18 Ab, the NF-κB inhibitor PDTC, the p38 inhibitor SB203580 and the ERK inhibitor PD98059, decidual iNKT cell percentages and CD69 expression in LPS-treated mice were markedly reduced when compared with controls ($P < 0.01$, respectively). However, pretreatment with the inhibitor of JNK, SP600125, had no effect on the decidual iNKT cell percentage or CD69 expression (Fig. 2).

![Figure 4](https://example.com/f4.png) **Figure 4** Comparison of decidual iNKT cell intracellular IL-4 levels following in vivo LPS exposure. C57BL/6 mice were injected i.p. with a panel of neutralizing Abs (or isotype control Abs) and inhibitors (or vehicle) in the presence or absence of LPS alone on Day 15 of gestation. Delivered mice were sacrificed and decidual MNCs were isolated. Decidual iNKT cell intracellular IL-4 production was analyzed using flow cytometry. (A) The percentages of iNKT cells positive for IL-4 in mice pretreated with neutralizing Abs are shown. Data are presented as mean ± SD of six independent experiments. (B) The percentages of iNKT cells positive for IL-4 in mice pretreated with inhibitors are presented. One representative experiment out of six is shown. Gray shaded histogram: isotype control. Black line: inhibitor treated. Numbers indicate the percentages of IL-4$^+$ cells among the iNKT cells (%). (C) A graphical summary of decidual iNKT cell intracellular IL-4 production in mice pretreated with inhibitors is presented. Data are presented as mean ± SD of six independent experiments.
Comparison of decidual iNKT cell intracellular cytokine production following *in vivo* LPS stimulation

*In vivo* LPS treatment promoted decidual iNKT cell activation in control mice as assessed by an increase in intracellular IFN-γ synthesis (*P* < 0.01) (Fig. 3). To further define the mechanisms behind this effect, we investigated whether enhanced intracellular IFN-γ production by decidual iNKT cells following *in vivo* LPS treatment was dependent on TLR4-mediated signals and CD1d expression. As expected, IFN-γ secretion was markedly reduced when LPS-exposed mice were pretreated with neutralizing Abs against TLR4, CD1d, IL-12 and IL-18, and inhibitors blocking NF-κB activation and p38 and ERK phosphorylation (*P* < 0.01, respectively) (Fig. 3). Consistent with our prior results, pretreatment with SP600125, which blocks the JNK pathway, failed to down-regulate IFN-γ release by decidual iNKT cells in response to *in vivo* LPS injection (Fig. 3B). In addition, *in vivo* treatment with LPS plus isotype control Abs and vehicle or LPS plus blocking Abs and inhibitors had no effects on decidual iNKT cell intracellular IL-4 production (Fig. 4).

Comparison of decidual iNKT cell intracellular cytokine levels and surface CD69 expression following *in vitro* LPS stimulation

To explore whether LPS-pulsed decidual DCs directly activate decidual iNKT cells, we used an *in vitro* co-culture system, in which decidual iNKT cells were co-cultured with LPS-sensitized decidual DCs or PBS-treated DCs. Confirming our *in vivo* intracellular cytokine data, LPS-pulsed DCs promoted decidual iNKT cell intracellular IFN-γ production compared with PBS-treated DCs (*P* < 0.01) (Fig. 5A and B). LPS-exposed DCs that were pretreated with Abs against TLR4, CD1d, IL-12 and IL-18, and inhibitors blocking the activation of NF-κB, p38 and ERK were

![Figure 5](image)

**Figure 5** Comparison of decidual iNKT cell intracellular IFN-γ and IL-4 production following *in vitro* LPS stimulation. Decidual DCs were incubated with neutralizing Abs (or isotype control Abs) and inhibitors (or vehicle), then treated with LPS or PBS for 12 h and extensively washed. A total of 2 × 10⁴ DCs were co-cultured with 2 × 10⁴ sorted decidual iNKT cells for 24 h. Decidual iNKT cell intracellular IFN-γ (**A** and **B**) and IL-4 secretion (**C** and **D**) were detected using flow cytometry. Data are presented as mean ± SD of six independent experiments. *P < 0.01, #P < 0.01 versus all the groups, except the SP600125 group; ΔP < 0.01 versus all the groups, except the DMSO+LPS group.
also less able to activate decidual iNKT cells to produce IFN-γ when compared with controls \( (P < 0.01, \text{respectively}) \) (Fig. 5A and B). In contrast, pretreatment of LPS-stimulated DCs with the JNK inhibitor, SP600125, did not affect their ability to activate decidual iNKT cells as shown by iNKT cell intracellular IFN-γ production (Fig. 5B). In addition, neither LPS plus isotype control Abs and vehicle nor LPS combined with the neutralizing Abs and inhibitors affected co-cultured iNKT cell intracellular IL-4 production (Fig. 5C and D).

**Comparison of decidual iNKT cell CD69 expression following in vitro LPS stimulation**

LPS-stimulated DCs significantly increased decidual iNKT cell CD69 expression compared with PBS-treated DCs \( (P < 0.01) \). Pretreatment of LPS-stimulated DCs with neutralizing Abs against TLR4, CD1d, IL-12 and IL-18, and inhibitors blocking NF-κB, p38 and ERK activation significantly down-regulated CD69 expression on decidual iNKT cells \( (P < 0.01, \text{respectively}) \). However, pretreatment of LPS-stimulated DCs with SP600125 did not change CD69 expression on decidual iNKT cells (Fig. 6).

**Comparison of decidual iNKT cell extracellular cytokine release following in vitro LPS stimulation**

As shown in Fig. 7A and B, co-culture of decidual iNKT cells with LPS-stimulated decidual DCs results in an increase in IFN-γ secretion into the supernatants \( (P < 0.01) \). LPS-exposed decidual DCs primarily produced IL-12 and IL-18 but not IFN-γ or IL-4 (Fig. 8). These data suggest that IFN-γ production in these co-cultures is dependent on iNKT cells but not DCs. In addition, IFN-γ release into the co-culture supernatants decreased substantially when LPS-pulsed DCs were pretreated with Abs against TLR4, CD1d, IL-12 and IL-18, and inhibitors blocking NF-κB activation, and p38 and ERK phosphorylation \( (P < 0.01, \text{respectively}) \) (Fig. 7A and B). Similar to our in vivo findings, pre-incubation of LPS-treated DCs with SP600125 had no effect on IFN-γ production in the co-culture supernatants (Fig. 7B). As expected, IL-4 levels in the co-culture supernatants did not change when DCs were treated with LPS plus isotype control Abs and vehicle or LPS plus the above-mentioned neutralizing Abs and inhibitors (Fig. 7C and D).

**Comparison of IFN-γ production in the culture supernatants of cytokine-treated decidual iNKT cells**

Neither IL-12 nor IL-18 alone, even at 1 ng/ml, could activate decidual iNKT cells to secrete significant levels of IFN-γ, regardless of the presence of anti-CD1d-treated DCs or isotype control Ab-treated DCs (Fig. 9A and B). However, 1 ng/ml IL-12 combined with serially diluted amounts of IL-18 induced IFN-γ secretion from decidual iNKT cells in the presence of isotype Ab-treated DCs but not anti-CD1d-treated DCs \( (P < 0.01, \text{respectively}) \) (Fig. 9C). A similar observation was made with IL-18 combined with serially diluted IL-12 \( (P < 0.01, \text{respectively}) \) (Fig. 9D).

**Discussion**

In an attempt to increase our understanding of the clinical problem of preterm birth, several animal models have been created. Intraperitoneal administration of inflammatory agents mimics maternal systemic infection and activates tumor necrosis factor (TNF)-α-dependent immune responses to induce preterm birth (Holmgren et al., 2008). In contrast, intrauterine injections that mimic uterine cavity infection do not tend

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**Figure 6** Comparison of decidual iNKT cell CD69 expression following in vitro LPS stimulation. Decidual DCs were incubated with neutralizing Abs (or isotype control Abs) and inhibitors (or vehicle), then treated with LPS or PBS for 12 h and extensively washed. A total of 2 × 10⁴ DCs were co-cultured with 2 × 10⁴ sorted decidual iNKT cells for 24 h. Decidual iNKT cell CD69 expression was measured using flow cytometry. (A) CD69 expression on decidual iNKT cells that are co-cultured with LPS-stimulated DCs pretreated with neutralizing Abs are shown. (B) CD69 expression on decidual iNKT cells that are co-cultured with LPS-stimulated DCs pretreated with inhibitors are presented. Data are presented as mean ± SD of six independent experiments. *P < 0.01; **P < 0.01 versus all the groups, except the SP600125 group; #P < 0.01 versus all the groups, except the DMSO+LPS group.
to activate a TNF-α-driven axis to induce preterm labor (Elovitz et al., 2003). Therefore, the same pathogen delivered through a different route can lead to activation of distinct inflammatory pathways. In addition, different pathogens may also elicit contrasting inflammatory responses. In our study, we have created a murine model of inflammation-induced preterm delivery by intraperitoneal injection of LPS. To model systemic endotoxemia (sepsis), we chose to administer LPS serotype O111:B4, a serotype of pathogenic enteric *E. coli* (Akarsu and Mamuk, 2007). The immune competence of adoptively transferred allogeneic iNKT cells has been demonstrated in various animal models (De Santo et al., 2008; Sada-Ovalle et al., 2008; Renneson et al., 2011; Lynch et al., 2012; Kim and Chung, 2013; Monteiro et al., 2013). In the present study, we employed decidual iNKT cell adoptive transfer to investigate the role of iNKT cells in LPS-induced preterm birth. The iNKT cells were adoptively transferred 2 h prior to LPS injection based on the observation that adoptive transfer of iNKT cells to *Jα18* KO mice results in the secretion of IFN-γ by iNKT cells that peaks at Day 1.5 after transfer (Wesley et al., 2008). Further, we have previously demonstrated that preterm delivery in mice occurs ~2 days after LPS injection (Li et al., 2012). Here, our data indicate that adoptive transfer of decidual iNKT cells into LPS-stimulated *Jα18* KO mice markedly decreased the time to delivery, suggesting an important role for iNKT cells in inflammation-associated preterm birth. However, donor decidual iNKT cells did not decrease the time to delivery in PBS-treated *Jα18* KO mice, indicating that the infusion of these allogeneic iNKT cells does not by itself explain increases in preterm birth in LPS-stimulated *Jα18* KO mice. Moreover, decidual iNKT cell adoptive transfer significantly increased the percentage of decidual iNKT cells in LPS-treated

**Figure 7** Comparison of decidual iNKT cell extracellular IFN-γ and IL-4 production following in vitro LPS stimulation. Decidual DCs were incubated with neutralizing Abs (or isotype control Abs) and inhibitors (or vehicle), then treated with LPS or PBS for 12 h and extensively washed. A total of 2 × 10⁴ DCs were co-cultured with 2 × 10⁴ sorted decidual iNKT cells for 24 h. Levels of IFN-γ (A and B) and IL-4 (C and D) in the culture supernatants were measured using ELISA. Data are presented as mean ± SD of six independent experiments. *P < 0.01; #P < 0.01 versus all the groups, except the SP600125 group; ∆P < 0.01 versus all the groups, except the DMSO + LPS group.
Ja18 KO mice, suggesting that donor decidual iNKT cell-mediated increase in preterm birth may occur through increasing the percentage of recipient decidual iNKT cells. However, iNKT cell adoptive transfer did not increase decidual iNKT cells in PBS-treated Ja18 KO mice. Our data suggest that LPS-induced systemic inflammation promotes release of chemotactic factors, which attract adopted iNKT cells to migrate to the decidua.

In vivo treatment with LPS significantly increased the percentage of decidual iNKT cells and their intracellular IFN-γ production and surface CD69 expression in C57BL/6 mice, suggesting that decidual iNKT cells can be activated by systemic LPS treatment. In vitro data indicated a potential mechanism for this finding since LPS-treated decidual DCs directly activated decidual iNKT cells, as assessed by elevated decidual iNKT cell intracellular and extracellular IFN-γ production and surface CD69 expression.

iNKT cells have been shown to play a critical protective role in host defense against a vast array of microbial pathogens, but the underlying mechanisms of iNKT cell activation by microbes are not fully understood. IL-12 and IL-18 have been implicated as being central to iNKT cell activation in response to TLR4-mediated stimulation of DCs in the absence of CD1d-presented microbial glycolipid antigens (Brigl et al.,...
Decidual iNKT cell activation promotes preterm birth

We utilized specific neutralizing Abs against IL-12 and IL-18 to measure their role in decidual iNKT cell activation. Our in vivo experiments revealed that treatment with blocking Abs against IL-12 or IL-18 dramatically decreased the percentages of decidual iNKT cells and their intracellular IFN-γ production and surface CD69 expression. Similarly, in vitro LPS-stimulated decidual DCs pretreated with anti-IL-12 and anti-IL-18 Abs significantly decreased decidual iNKT cell intracellular and extracellular IFN-γ production and surface CD69 expression. These data indicate that decidual iNKT cell activation in response to LPS stimulation is IL-12- and IL-18-dependent. Whether IL-12 or IL-18 alone is sufficient for decidual iNKT cell activation is unknown. To clarify this, IFN-γ production by purified decidual iNKT cells was measured after treatment with various amounts of IL-12, IL-18, or IL-12 and IL-18 in the presence of anti-CD1d-treated DCs or isotype control Ab-treated DCs. IL-12 or IL-18 alone could not induce IFN-γ secretion from iNKT cells, regardless of the dose used, whereas IL-12 combined with IL-18 could promote IFN-γ secretion by iNKT cells in the presence of isotype control Ab-treated DCs but not anti-CD1d-treated DCs. Our data demonstrate that IL-12 and IL-18 are each necessary but not sufficient for decidual iNKT cells activation, whereas IL-12 can synergize with IL-18 to enhance decidual iNKT cell IFN-γ production. In addition, our data also suggest that decidual iNKT cell activation is also dependent on CD1d expression on DCs.

The secretion of IL-12 and IL-18 in response to LPS stimulation relies on TLR4 MyD88-dependent pathways (Yang et al., 2011; Kondo et al., 2012; Xu et al., 2012; Bonham et al., 2014). Prior studies have assessed the role of TLR-mediated upstream pathways in iNKT cell activation using DCs deficient in TLR9 (Paget et al., 2007) or MyD88 (Brigl and Brenner, 2010; Brigl et al., 2011). In order to investigate more detailed molecular mechanisms of decidual iNKT cell activation, we used various blocking Abs and inhibitors targeting the key components of MyD88-dependent TLR4 signaling pathways from ligand engagement to downstream signaling events. Neutralizing Ab against TLR4 and inhibitors blocking NF-κB, MAPK p38 and ERK activation significantly reduced in vivo decidual iNKT cell percentages, intracellular IFN-γ production and surface CD69 expression. In vitro decidual iNKT cell extracellular and intracellular IFN-γ secretion and surface CD69 expression in response to LPS stimulation were also reduced. Interestingly, we did not detect any appreciable effect of JNK blockade on decidual iNKT cell activation using SP600125, a specific JNK inhibitor. Taken together, our data demonstrate that TLR4-mediated NF-κB, p38 and ERK pathways, and IL-12 and IL-18 secretion are essential for decidual iNKT cell activation induced by LPS from E. coli. Our data confirm prior reports showing that TLR4 activation in DCs results in iNKT cell activation (Brigl et al., 2003; Mattner et al., 2005; Nagarajan and Kronenberg, 2007). In addition, we clarify the TLR4 signaling pathways that are indispensable for decidual iNKT cell activation.

It is controversial whether indirect activation of iNKT cells requires TCR-mediated signals via recognition of CD1d-presented endogenous glycolipids. It is known that the semi-invariant TCR of iNKT cells specifically recognizes a range of endogenous and exogenous glycolipid antigens in the context of CD1d (Venkataswamy and Porcelli, 2010). In our study, anti-CD1d Ab treatment decreased the percentage of decidual iNKT cells, their intracellular and extracellular IFN-γ production and their surface CD69 expression upon LPS stimulation in the absence of exogenous antigens, suggesting that the activation of decidual iNKT cells is also dependent on TCR stimulation by endogenous glycolipids presented by CD1d. This finding is consistent with studies reporting that iNKT cell activation requires the recognition of CD1d-presented self-antigens (Brigl et al., 2003; Mattner et al., 2005), but differs from a report that demonstrates that IL-12 and IL-18 can directly activate iNKT cells in a TCR-independent manner upon stimulation with LPS from E. coli and Salmonella (Nagarajan and Kronenberg, 2007). The reasons for this discrepancy are not known, but could be related to the organ-specific and heterogeneous phenotypes of iNKT cells, differences in tissue milieus, discrepancies in the sources and doses of LPS, or differences in the methods used to prepare DCs for in vitro experiments. Whether one or more of these is responsible, the divergent results emphasize the existence of multiple pathways that can lead to the indirect activation of iNKT cells.

LPS activates TLR4-expressing DCs and rapidly induces IL-12 and IL-18 production (Nagarajan and Kronenberg, 2007). These proinflammatory cytokines then activate early IFN-γ production from iNKT cells. IFN-γ produced by iNKT cells feeds back on DCs, together with CD40-CD40L interactions, and further leads to potent activation of DCs (Van Kaer et al., 2011). Enhanced IFN-γ secretion from activated iNKT cells can promote the activation of other immune cells in the innate and adaptive immune systems, including NK cells, T cells, and neutrophils (Brennan et al., 2013), which can further amplify inflammatory responses at the maternal-fetal interface and ultimately results in preterm birth. We have previously shown that iNKT cells promote inflammation-induced preterm delivery by activating decidual DCs, NK cells and T cells (Li et al., 2012).

In summary, our data demonstrate that decidual iNKT cells can be activated in response to LPS stimulation by TLR4-mediated pathways and IL-12 and IL-18 secretion, in combination with TCR-mediated responses to endogenous glycolipids presented by CD1d. The activation of iNKT cells at the maternal-fetal interface plays a key role in inflammation-induced preterm birth. The indirect pathway of activation enables decidual iNKT cells to respond rapidly to a wide variety of microbes despite a limited TCR diversity, and contributes to inflammatory reactions at the maternal-fetal interface. Our findings provide a better understanding of the molecular mechanisms of iNKT cell activation during microbial infection and the role of iNKT cells in inflammation-induced preterm birth. The development of iNKT cell-based immunotherapies that target specific pathways of iNKT cell activation holds great promise for the treatment of iNKT cell-associated inflammatory diseases, including preterm birth.

Authors’ roles

L.L. and D.J.S. conceived and designed the study. J.Y., Y.J. and J.T. performed the experiments. L.L. performed the data analysis and wrote the draft manuscript. D.J.S. revised the manuscript. All authors contributed to the final version of the manuscript.

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**Conflict of interest**

The authors declare no commercial or financial conflict of interest.

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