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Depletion of Invariant NKT Cells Reduces Inflammation-Induced Preterm Delivery in Mice

Li-Ping Li,* Yi-Chuan Fang,* Guo-Fa Dong,* Yi Lin,† and Shigeru Saito‡

This study sought to determine whether invariant NKT (iNKT) cells play an essential role in inflammation-induced preterm delivery. Preterm delivery and fetal death rates were determined in wild-type (WT) C57BL/6 mice and iNKT cell-deficient Jα18$^{-/-}$ mice injected i.p. with LPS. The percentages of decidual immune cells, including activated subsets, and costimulatory molecule expression were analyzed by flow cytometry. Th1 and Th2 cytokine production in the culture supernatants of decidual mononuclear cells was measured by ELISA. To some extent, Jα18$^{-/-}$ mice were resistant to LPS-induced preterm delivery. The proportions of decidual CD3$^+$ and CD49b$^+$ cells were slightly lower in Jα18$^{-/-}$ mice than in WT Jα18$^{+/+}$ mice, whereas almost no CD3$^+$CD49b$^+$ cells could be found in Jα18-null mice. The percentages of activated decidual DCs, T cells, and NK cells were significantly lower in LPS-treated Jα18$^{-/-}$ mice than in WT mice. The CD40, CD80, and CD86 expression levels on decidual CD11c$^+$ cells from Jα18$^{-/-}$ mice were also significantly lower than in WT mice. Mean concentrations of Th1 cytokines IFN-γ and IL-12p70 in the culture supernatants of decidual mononuclear cells from LPS-treated Jα18$^{-/-}$ mice were apparently lower than those of LPS-induced WT mice. Additionally, the proportions of activated CD11c$^+$ cells, CD3$^+$ cells, and CD49b$^+$ cells in LPS-induced preterm delivery mice were strikingly higher in both WT and null mice when compared with the control PBS group and LPS-injected but normally delivered mice. Our results suggest that iNKT cells may play an essential role in inflammation-induced preterm birth. ***The Journal of Immunology*****, 2012, 188: 4681–4689.**

Preterm birth, defined as childbirth occurring at ˂37 completed weeks or 259 d gestation, occurs in 9.6% of all births worldwide (1). Preterm birth is recognized as a worldwide problem, accounting for ˃80% of neonatal deaths and ˃50% of long-term morbidity in the surviving infants (2). Among surviving infants, preterm birth is implicated by long-term neurodevelopmental disabilities, mental retardation, and cognitive, executive, and educational deficits (3–5).

The most common cause of preterm birth is infection, which is largely subclinical in nature (6). Up to 30% of cases of spontaneous preterm birth are associated with intrauterine microbial colonization or histological evidence of chorioamnionitis. Intraamniotic inflammation is found in approximately half of the cases of preterm labor and premature preterm rupture of membranes (7). The presence of intrauterine inflammation is not only a cause of preterm birth but is also associated with an increase in neonatal morbidity and mortality.

TLRs are a group of pattern-recognition receptors that recognize conserved microbial structures called pathogen-associated molecular patterns on the surface of bacteria, viruses, fungi, and protozoans. TLRs play an essential role in immune recognition, cell signal transduction, upstream events of immune response cascades, and inflammation-induced preterm birth (8–10). Despite a growing association between TLRs and inflammation-induced preterm birth, the precise mechanisms of TLR-mediated inflammation at the maternal/fetal interface are largely unknown.

NKT cells are a subset of innate lymphocytes that were originally identified as an unusual T cell population that coexpress TCRs and receptors of the NK cell lineage. Most NKT cells express a semi-invariant TCR, Vα14-Jα18/Vβ8.2, Vβ7, or Vβ2 in mice and Vα24-Jα18/Vβ11 in humans (11). As such, these cells have a limited ligand repertoire and are referred to as invariant NKT (iNKT) cells or type I NKT cells. In sharp contrast with conventional T cells, which recognize peptide Ags presented by MHC class I or II proteins, iNKT cells are specific for glycolipid Ags presented by the MHC class I-related protein CD1d (12).

NKT cells are different from functionally differentiated conventional αβ T cells in that they are autoreactive and swiftly produce copious amounts of both Th1 and Th2 cytokines, including IL-4, IL-10, and IFN-γ, upon stimulation with their ligands (12). The cytokines secreted by activated iNKT cells amplify the immune response initiated by these cells through transactivation of other cell types, including conventional CD4 and CD8 T cells, dendritic cells (DCs), NK cells, B cells, regulatory T cells, macrophages, and neutrophils (11), and they influence a broad spectrum of immunological responses, including autoimmunity (13), antimicrobial host responses (14), resistance to tumors (15), and transplantation immunity (16).

It is reported that activation of human APCs by TLR ligands modulates the lipid biosynthetic pathway, resulting in enhanced recognition of CD1d-associated lipids by iNKT cells, as defined by

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Abbreviations used in this article: DC, dendritic cell; iNKT cell, invariant NKT cell; WT, wild-type.

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IFN-γ secretion (17). APC-derived soluble factors further increase CD1d-restricted iNKT cell activation (17). Therefore, iNKT cells at the maternal/fetal interface may be activated by self-lipid Ags versus soluble factors from DCs upon TLR signaling, and play a role in inflammation-induced preterm birth. As we have previously demonstrated that TLR4 plays an important role in LPS-induced preterm birth (18), we wanted to assess in this study the impact of iNKT cells on TLR4-induced inflammatory preterm birth.

Conventional T cells do not express the invariant Vα14-Jα18 Ag receptor (19), 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 lymphoid cells intact, including T, B, and NK cells (20, 21). Jα18$^{-/-}$ mice were found to be fertile and are healthy in appearance (22). The number of total lymphocytes in Jα18$^{-/-}$ mice are not significantly different from Jα18$^{+/+}$ mice, with the exception of a complete loss of Vα14 NKT cells (20, 21). Ito et al. (21) have described the possible role of decidual Vα14 NKT cells in the pathogenesis of abortion. However, it remains unclear whether iNKT cells play a key role in the pathogenesis of preterm labor. In our present study, we made use of Jα18$^{-/-}$ mice to investigate the possible role that iNKT cells may play in inflammation-induced preterm birth.

Materials and Methods

Animals

Eight-week-old female and male wild-type (WT) C57BL/6 mice and iNKT cell-deficient Jα18$^{-/-}$ mice on a C57BL/6 background were purchased from The Jackson Laboratory and subsequently maintained under pathogen-free conditions in the Laboratory Animal Facility of Guangzhou Medical College. Animals were acclimated in our facility for at least 2 wk before use in these experiments. All animal procedures followed the guidelines of the Chinese Council for Animal Care. Each female mouse was co-caged with one male. The point at which a vaginal plug was detected was designated as day 0 gestation.

LPS-induced preterm delivery model

As described previously (18), on day 15 gestation, the pregnant Jα18$^{-/-}$ mice or C57BL/6 mice were injected i.p. with 200 μl LPS (Sigma-Aldrich) saline solution with care not to enter the amniotic cavity. The dose of LPS was generally 50 μg/kg body weight, unless otherwise indicated. Pregnant WT mice and Jα18$^{-/-}$ mice injected i.p. with 200 μl PBS served as negative controls. Animals were observed closely for any signs of morbidity (piloerection, decreased movement, vaginal bleeding, and preterm delivery). Mice that delivered prematurely or delivered at term were autopsied when fetuses were found in the cage. Preterm delivery was defined as the finding of at least one pup in the cage or the lower vagina before gestation day 18 (not including gestation day 18). Fetal death was identified by white discoloration, markedly smaller fetal size, and lack of blood flow in the umbilical cord. The incidences of preterm delivery and fetal death were calculated. Preterm delivery rate was determined by total prematurely delivered mice/total pregnant mice. Fetal death rate was determined by total dead fetuses/total fetuses. Because the maternal mice may cannibalize dead pups before being counted, the fetal death rate may be underestimated. To minimize this possibility, mice were monitored frequently after PBS or LPS injection (no less than four times a day at an interval of <8 h).

Isolation of peripheral blood and decidual mononuclear cells

Peripheral blood samples were collected from the vena orbitalis, heparinized, and purified by centrifugation on Ficoll-Hypaque density medium (23). For decidual tissue harvest, the uterine horns of pregnant mice were opened longitudinally, and the whole placental and decidual unit was separated individually from the respective embryo and its implantation site. After washing in cold PBS, pooled decidual tissues were cut into small pieces (1 mm^3). To minimize the underestimation of immunological changes, samples from mice that gave live births or appeared healthy during gestation were separated from those with adverse outcomes in the same group. Only the samples from those with adverse outcomes in the same group were pooled, analyzed, and compared with the control group, unless otherwise indicated. The selected tissue was digested three times with 1 mg/ml Dispase II (Roche) at 37°C for 20 min in each cycle in a shaking water bath. When single or clumps of cells were observed under a microscope, the released cells were separated from undigested tissue pieces by filtering through a 50-μm-pore nylon mesh. Mononuclear cells were purified with Ficoll-Hypaque density medium (density, 1.077 ± 0.002 g/ml) by centrifugation at 800 × *g* for 20 min at 22°C. Any contaminating RBCs that might have persisted in peripheral blood or decidual single-cell suspension were eliminated by incubation with red cell lysis buffer (GenMed) (23).

Flow cytometry analysis

Every 10^6 PBMCs or decidual mononuclear cells in 50 μl PBS were incubated with PE/Cy5-conjugated CD45 (0.25 μg), PE-conjugated CD11c (0.25 μg), FITC-conjugated CD3 (0.5 μg), allophycocyanin/Cy7-conjugated CD19 (0.5 μg), allophycocyanin-conjugated CD49b (0.5 μg), and PE/Cy7-conjugated CD69 (0.25 μg) for 30 min at 4°C. In some cases, every 10^6 decidual mononuclear cells in 50 μl PBS were incubated with PE/Cy5-conjugated CD45 (0.25 μg), PE-conjugated CD11c (0.25 μg), FITC-conjugated CD40 (0.5 μg), allophycocyanin-conjugated CD80 (0.5 μg), and PE/Cy7-conjugated CD86 (0.5 μg) for 30 min at 4°C. After washing twice with PBS, cells were fixed in 10 g/l paraformaldehyde. Immunostained cells were analyzed on a FACSCanto flow cytometer using FACSDiva software (Becton Dickinson). Ten thousand cells were detected in each sample. Isotype controls were established using matched fluorescence-labeled isotype control Abs to avoid nonspecific staining. All of the fluorescence-labeled Abs and isotype controls were purchased from BioLegend.

The percentage of decidual CD11c$^+$ cells in the CD45$^+$ cell population was calculated as follows: % CD11c$^+$ cells in CD45$^+$ cell population = (CD11c$^+$CD45$^+$ cell number/CD45$^+$ cell number) × 100. In the same way, the percentages of decidual CD3$^+$ cells in the CD45$^+$ cell population, CD19$^+$ cells in the CD45$^+$ cell population, CD49b$^+$ cells in the CD45$^+$ cell population, CD11c$^+$CD69$^+$ cells in the CD11c$^+$ cell population, CD3$^+$CD69$^+$ cells in the CD3$^+$ cell population, CD19$^+$CD69$^+$ cells in the CD19$^+$ cell population, and CD49b$^+$CD69$^+$ cells in the CD49b$^+$ cell population were calculated. In other cases, the percentages of decidual CD11c$^+$CD40$^+$ cells in the CD11c$^+$ cell population, CD11c$^+$CD80$^+$ cells in the CD11c$^+$ cell population, and CD11c$^+$CD86$^+$ cells in the CD11c$^+$ cell population were calculated in the same way.

Decidual mononuclear cells harvested on gestation day 15 were isolated by density centrifugation and further purified using MACS microbead-conjugated anti-mouse CD3 and anti-mouse CD49b (Miltenyi Biotec) to obtain the indicated cell subsets. Cells were counted using a hemocytometer and compared between untreated Jα18$^{+/+}$ and Jα18$^{-/-}$ mice. The experiments were performed independently four times.

Cytokine assays

Decidual mononuclear cells were cultured in a 5% CO_2 atmosphere. After 24 h, culture supernatants were collected and stored at −80°C for cytokine examination. The levels of mouse cytokines, including IFN-γ, IL-12p70, IL-4, and IL-10, were assessed using commercially available ELISA kits from BD Pharmingen. All assays were conducted according to the manufacturer's instructions.

Statistical analysis

Preterm delivery rates were examined using a Fisher exact test, and fetal death rates were analyzed using the Pearson χ^2 text. Gestation length, cell percentages, and cytokine concentrations were determined by one-way ANOVA. Results were given as means ± SD. A p value of <0.05 was considered significant between analyzed groups.

Results

Comparison of gestation length between pregnant Jα18$^{+/+}$ mice and Jα18$^{-/-}$ mice

C57BL/6 mice normally deliver pups from day 19 to 21 of gestation. To determine whether iNKT cell deficiency influences the gestation length of normal labor, gestation lengths for PBS-treated Jα18$^{+/+}$ and PBS-treated Jα18$^{-/-}$ mice were compared. As shown in Fig. 1, the mean gestation length for iNKT cell-deficient Jα18$^{-/-}$ mice treated with PBS was 19.8 ± 0.8 d, which was not significantly different from that for Jα18$^{+/+}$ mice (20.0 ± 0.7 d). No

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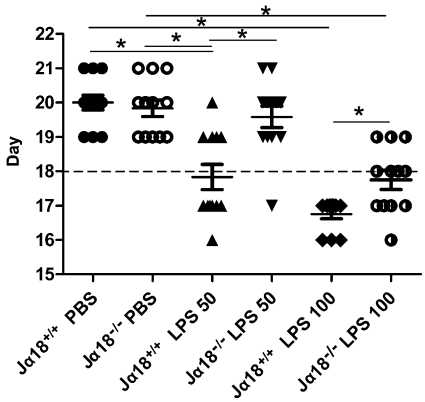

FIGURE 1. Comparison of gestation lengths between $J\alpha18^{+/+}$ and $J\alpha18^{-/-}$ mice. $J\alpha18^{+/+}$ and $J\alpha18^{-/-}$ mice were treated with PBS, or LPS at a dosage of 50 μg/kg body weight (LPS 50), or LPS at a dosage of 100 μg/kg body weight (LPS 100). *$p < 0.01$. Each spot below the dashed line indicates one preterm labor mouse.

labor delay was found in $J\alpha18^{-/-}$ mice. In comparison, the mean gestation length was significantly shorter in LPS-treated WT ($J\alpha18^{+/+}$) mice at a dosage of 50 μg/kg body weight than $J\alpha18^{-/-}$ mice with the same treatment (17.8 ± 1.3 versus 19.6 ± 1.1 d; $p = 0.001$). When the mice were treated with LPS at a dosage of 100 μg/kg body weight, a significantly decreased gestation length was observed in both WT and $J\alpha18^{-/-}$ mice (16.8 ± 0.5 versus 17.8 ± 1.0 d; $p = 0.004$). Thus, the LPS dosage of 50 μg/kg body weight was generally used in the present study (Fig. 1).

Comparison of preterm delivery rate and mortality

To access the intrinsic role of iNKT cells in inflammation-induced preterm delivery, the LPS-induced preterm delivery model was established in $J\alpha18^{+/+}$ mice and $J\alpha18^{-/-}$ mice, and the rates of preterm delivery and mortality were analyzed. In our model, i.p. injection of LPS at 50 μg/kg body weight resulted in a 58.3% preterm birth rate in $J\alpha18^{+/+}$ mice within 72 h, which was markedly higher than that of PBS-treated WT mice and PBS-treated $J\alpha18^{-/-}$ mice whose preterm birth rates were both zero ($p < 0.01$). All preterm delivered maternal mice delivered nonviable pups, and no maternal mortality or morbidity was observed. The incidence of preterm birth was significantly decreased in LPS-injected $J\alpha18^{-/-}$ mice (group D) compared with LPS-injected $J\alpha18^{+/+}$ mice (group C) ($p < 0.05$) (Table I).

Although the LPS dose of 100 μg/kg body weight caused a 100% preterm birth rate in WT mice, we still did not observe maternal mortality in this model. The incidence of preterm labor was markedly downregulated in LPS-treated $J\alpha18^{-/-}$ mice (group F) compared with LPS-treated $J\alpha18^{+/+}$ mice (group E) ($p < 0.01$), although it was still significantly higher than that of PBS-treated $J\alpha18^{+/+}$ mice (group A) ($p < 0.05$) or $J\alpha18^{-/-}$ mice (group B) ($p < 0.05$) (Table I).

In LPS-treated WT mice at a dosage of 50 μg/kg body weight, intrauterine demise was observed in 57.6% of fetuses, which was significantly higher than that found in PBS-treated $J\alpha18^{+/+}$ mice and PBS-treated $J\alpha18^{-/-}$ mice (fetal death rate of 3.0 and 3.8%, respectively; $p < 0.01$). Meanwhile, the mortality in LPS-treated $J\alpha18^{-/-}$ mice was markedly lower than in LPS-treated $J\alpha18^{+/+}$ mice ($p < 0.01$) (Table I).

A higher dosage of LPS (100 μg/kg) resulted in a 100% fetal death in WT mice. The incidence of fetal death was markedly lower in LPS-treated $J\alpha18^{-/-}$ mice (group F) than in LPS-treated $J\alpha18^{+/+}$ mice (group E) ($p < 0.01$), although it was still significantly higher than in PBS-injected $J\alpha18^{+/+}$ and $J\alpha18^{-/-}$ mice ($p < 0.01$ for both) (Table I). It is notable that the extent of fetal death reported in this study is probably inaccurate and likely to be underestimated, as the mother mice commonly cannibalize dead pups.

Comparison of the percentages of peripheral blood and decidual DC, T cell, B cell, and NK cell populations

To explore the mechanism underlying the decreased preterm delivery and fetal death rates in iNKT cell-deficient $J\alpha18^{-/-}$ mice, we examined the percentages of decidual DCs, T cells, B cells, and NK cells by detecting their respective surface markers CD11c, CD3, CD19, and CD49b. We found no significant difference in the percentages of decidual DCs and B cells between PBS-treated $J\alpha18^{+/+}$ and $J\alpha18^{-/-}$ mice. However, the percentages of decidual $CD3^+$ cells and $CD49b^+$ cells were slightly higher in $J\alpha18^{+/+}$ mice than in $J\alpha18^{-/-}$ mice. The percentages of decidual $CD3^+$ cells and $CD49b^+$ cells from LPS-injected $J\alpha18^{+/+}$ mice were significantly higher than those from PBS-injected $J\alpha18^{+/+}$ mice and $J\alpha18^{-/-}$ mice. Additionally, the percentages of decidual T cells and NK cells from LPS-injected $J\alpha18^{-/-}$ mice were markedly lower than those for LPS-treated $J\alpha18^{+/+}$ mice. Moreover, in peripheral blood from nonpregnant and pregnant mice, the numbers of DCs, T cells, B cells, and NK cells were similar among the four groups (Fig. 2).

Table I. Comparison of preterm delivery and mortality rates

Group	Mice	LPS	*n*	Preterm Delivery Rate	Mortality
A	$J\alpha18^{+/+}$	PBS	12	0 (0/12)	3.0% (3/101)
B	$J\alpha18^{-/-}$	PBS	12	0 (0/12)	3.8% (4/105)
C	$J\alpha18^{+/+}$	50 μg/kg	12	58.3% (7/12)[a,b]	57.6% (57/99)[a,b]
D	$J\alpha18^{-/-}$	50 μg/kg	12	8.3% (1/12)[c]	8.8% (9/102)[d]
E	$J\alpha18^{+/+}$	100 μg/kg	12	100% (12/12)[a,b]	100% (104/104)[a,b]
F	$J\alpha18^{-/-}$	100 μg/kg	12	41.7% (5/12)[e,f,g]	38.3% (41/107)[a,b,g]

On day 15 gestation, pregnant $J\alpha18^{-/-}$ and C57BL/6 mice were i.p. injected with 200 μl PBS or LPS at 50 or 100 μg/kg body weight. Mice that delivered preterm or delivered at term were autopsied when fetuses were found in the cage. The incidences of preterm delivery and fetal death were calculated. Preterm delivery rate = total preterm delivered mice/total pregnant mice. Fetal death rate = total dead fetuses/total fetuses.

[a] $p < 0.01$ versus group A.
[b] $p < 0.01$ versus group B.
[c] $p < 0.05$ versus group C.
[d] $p < 0.01$ versus group C.
[e] $p < 0.05$ versus group A.
[f] $p < 0.05$ versus group B.
[g] $p < 0.01$ versus group E.

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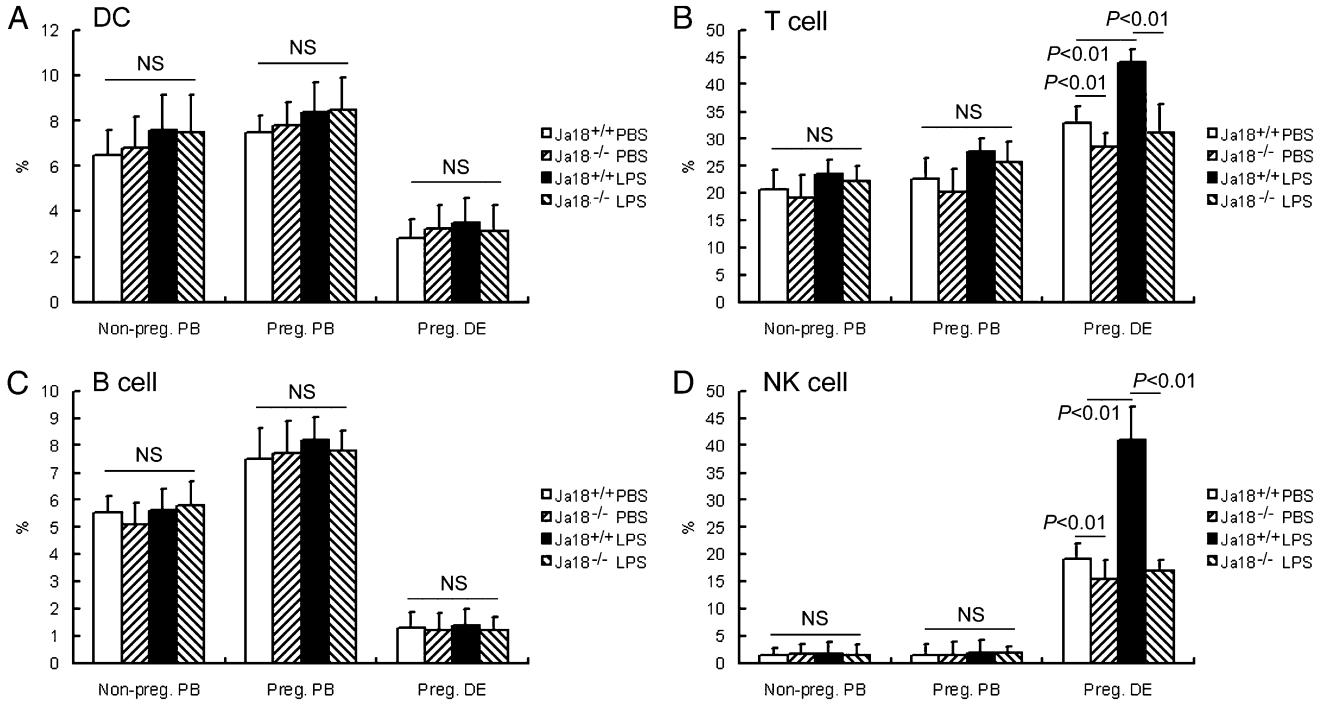

FIGURE 2. Percentages of peripheral blood and decidual DC (**A**), T cell (**B**), B cell (**C**), and NK cell (**D**) populations in nonpregnant and pregnant mice. Pregnant (Preg.) J$\alpha18^{-/-}$ mice or J$\alpha18^{+/+}$ mice were injected i.p. with PBS or LPS at 50 μg/kg body weight. PBMCs from nonpregnant mice and peripheral blood and decidual mononuclear cells from pregnant mice were isolated and incubated with fluorescence-conjugated anti-CD45, anti-CD11c, anti-CD3, anti-CD19, and anti-49b mAbs and isotype controls. The percentages of $CD11c^+$ cells, $CD3^+$ cells, $CD19^+$ cells, and $CD49b^+$ cells in the $CD45^+$ cells were calculated. Values represent means ± SD (n = 4 for each group).

Comparison of the absolute number of decidual $CD3^+$, $CD49b^+$, $CD3^+CD49b^-$, $CD49b^+CD3^-$, and $CD3^+CD49b^+$ cells

Absolute numbers of MACS-purified decidual $CD3^+$, $CD49b^+$, $CD3^+CD49b^-$, $CD49b^+CD3^-$, and $CD3^+CD49b^+$ cells were compared between untreated J$\alpha18^{+/+}$ mice and J$\alpha18^{-/-}$ mice, and the representative flow cytometry results are shown in Fig. 3A. Whereas almost no $CD3^+CD49b^+$ cells were found in J$\alpha18^{-/-}$ mice (0.8×10^4), a considerable number of the double-positive cells were observed in J$\alpha18^{+/+}$ mice (9.0×10^4; $p < 0.01$) (Fig. 3B). Both $CD3^+$ and $CD49b^+$ cell numbers were slightly higher in

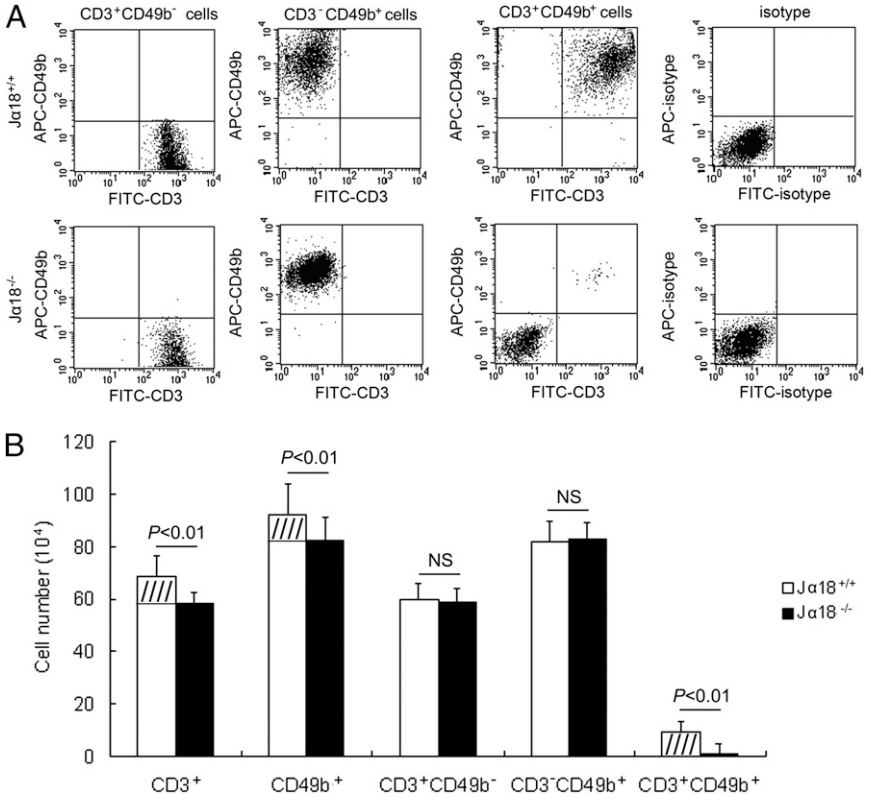

FIGURE 3. Comparison of the absolute number of decidual cell subsets between J$\alpha18^{+/+}$ and J$\alpha18^{-/-}$ mice. MACS-purified cell subsets were compared. (**A**) MACS-purified cells analyzed by flow cytometry. Cell purity routinely exceeded 97%. (**B**) Comparison of absolute cell numbers of indicated cell subsets. Results are shown as means ± SD (n = 4 for each group). Values of the shadowed bars are similar between the three indicated groups.

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WT mice than in Jα18$^{-/-}$ mice (1.2-fold for CD3$^+$ cells and 1.1-fold for CD49b$^+$ cells; $p < 0.01$ for both), but no significant difference was found in the number of CD3$^+$CD49b$^-$ or CD49b$^+$ CD3$^-$ cells between Jα18$^{+/+}$ and Jα18$^{-/-}$ mice. In the WT mice the number of CD3$^+$CD49b$^-$ cells plus the number of CD3$^+$ CD49b$^+$ cells appeared approximately equal to the number of CD3$^+$ cells, and the number of CD49b$^+$CD3$^-$ cells plus the number of CD3$^+$CD49b$^+$ cells appeared approximately equal to the number of CD49b$^+$ cells, suggesting that the decreased decidual CD3$^+$ and CD49b$^+$ cell numbers in the Jα18$^{-/-}$ mice may be completely attributed to the absence of iNKT cells (Fig. 3B).

Comparison of the percentages of activated decidual DC, T cell, B cell, and NK cell populations

We also wanted to determine whether iNKT cells affect lymphocyte activation at the maternal/fetal interface. The early activation marker CD69 on decidual DCs, T cells, B cells, and NK cells were analyzed by flow cytometry. There was no statistically supported difference in the percentages of decidual activated DCs, T cells, B cells, or NK cells between PBS-treated Jα18$^{+/+}$ mice and PBS-treated Jα18$^{-/-}$ mice. At the dose of 50 μg/kg body weight, LPS-injected Jα18$^{+/+}$ mice showed markedly increased percentages of decidual activated DCs, T cells, and NK cells in comparison with PBS-injected Jα18$^{+/+}$ or Jα18$^{-/-}$ mice. The percentages of decidual activated DCs, T cells, and NK cells from LPS-injected Jα18$^{-/-}$ mice were strikingly reduced when compared with those from LPS-injected Jα18$^{+/+}$ mice.

At the dose of 100 μg/kg body weight, LPS-injected WT mice showed significantly increased percentages of decidual activated DCs, T cells, and NK cells in comparison with LPS-infused WT mice at the dose of 50 μg/kg. At the dose of 100 μg/kg, the percentages of activated decidual DCs, T cells, and NK cells in LPS-treated Jα18$^{-/-}$ mice were markedly downregulated when compared with LPS-injected WT mice; however, they were still significantly higher than those in LPS-treated Jα18$^{-/-}$ mice at the dose of 50 μg/kg and in the two control PBS groups (Table II).

Comparison of CD40, CD80, and CD86 expression on decidual DCs

To further explore the possible impact of iNKT cells on decidual DCs, we evaluated the expression of costimulatory molecules CD40, CD80, and CD86 on decidual DCs from Jα18$^{-/-}$ mice and WT mice. There were no significant differences in the CD40, CD80, and CD86 expression levels on decidual DCs between PBS-treated Jα18$^{+/+}$ mice and PBS-treated Jα18$^{-/-}$ mice. The percentages of decidual CD40$^+$, CD80$^+$ and CD86$^+$ cells in the CD11c$^+$ cell population from LPS-injected Jα18$^{+/+}$ mice were significantly higher than those from PBS-injected Jα18$^{+/+}$ and Jα18$^{-/-}$ mice. The percentages of decidual CD40$^+$, CD80$^+$, and CD86$^+$ cells in the CD11c$^+$ population from LPS-treated Jα18$^{-/-}$ mice were significantly lower than those in LPS-treated Jα18$^{+/+}$ mice (Table III).

Analysis of cytokines in the culture supernatants of decidual mononuclear cells

To better understand the mechanism by which iNKT cells affect LPS-induced preterm delivery and fetal death, the levels of Th1 and Th2 cytokines in the culture supernatants of decidual mononuclear cells from Jα18$^{-/-}$ mice and WT mice were investigated. No significant differences were observed in the concentrations of IFN-γ, IL-12p70, IL-4, and IL-10 between PBS-treated Jα18$^{+/+}$ mice and PBS-treated Jα18$^{-/-}$ mice. However, the concentrations of Th1 cytokines IFN-γ and IL-12p70 in the culture supernatants of decidual mononuclear cells from LPS-injected WT mice were apparently upregulated when compared with the two control PBS groups. The concentrations of IFN-γ and IL-12p70 in the culture supernatants were significantly lower in LPS-treated Jα18$^{-/-}$ mice than in LPS-treated Jα18$^{+/+}$ mice. In contrast, the concentrations of Th2 cytokines IL-4 and IL-10 in the supernatants from LPS-treated Jα18$^{-/-}$ mice showed no difference from those of LPS-treated Jα18$^{+/+}$ mice (Fig. 4).

Comparison of the percentages of activated decidual DC, T cell, B cell, and NK cell populations between preterm delivered mice and normally delivered mice

To further investigate the mechanisms of preterm delivery, we pooled the decidua of LPS-induced preterm delivered Jα18$^{+/+}$ mice and Jα18$^{-/-}$ mice and pooled the decidua of LPS-injected but normally delivered Jα18$^{+/+}$ mice and Jα18$^{-/-}$ mice ($n = 4$ for each group). We compared the percentages of activated DCs, T cells, B cells, and NK cells from LPS-induced preterm delivered WT and null mice with those from LPS-treated but normally delivered WT and null mice. PBS-treated WT and null mice were used as controls. There were no substantial differences in the percentages of decidual DCs, T cells, B cells, and NK cells among the LPS-injected but normally delivered mice, in either Jα18$^{+/+}$ mice or Jα18$^{-/-}$ mice. The percentages of activated decidual DCs, T cells, and NK cells from LPS-induced preterm labor mice were increased to a significantly higher level than those from the control PBS groups and LPS-treated but normally delivered Jα18$^{+/+}$ and Jα18$^{-/-}$ mice (Fig. 5).

Table II. Comparison of the percentages of activated decidual DC, T cell, B cell, and NK cell populations

Group	Mice	LPS	Activated DCs	Activated T Cells	Activated B Cells	Activated NK Cells
A	Jα18$^{+/+}$	PBS	9.3 ± 1.5	9.0 ± 1.4	6.4 ± 0.9	7.2 ± 1.3
B	Jα18$^{-/-}$	PBS	9.5 ± 1.3	8.8 ± 1.5	5.8 ± 0.6	6.9 ± 1.1
C	Jα18$^{+/+}$	50 μg/kg	25.4 ± 2.7^{a,b}	20.5 ± 2.4^{a,b}	6.1 ± 1.1	26.9 ± 2.8^{a,b}
D	Jα18$^{-/-}$	50 μg/kg	9.3 ± 1.7^{c}	9.1 ± 1.4^{c}	5.9 ± 0.9	7.3 ± 1.0^{c}
E	Jα18$^{+/+}$	100 μg/kg	39.4 ± 4.1^{a,b,c}	49.3 ± 5.1^{a,b,c}	6.8 ± 1.0	61.5 ± 5.7^{a,b,c}
F	Jα18$^{-/-}$	100 μg/kg	19.3 ± 2.9^{a,b,d,e}	18.5 ± 2.6^{a,b,d,e}	6.6 ± 0.8	22.2 ± 2.7^{a,b,d,e}

Pregnant Jα18$^{+/+}$ and Jα18$^{-/-}$ mice were i.p. injected with PBS or LPS (50 or 100 μg/kg body weight). In flow cytometric analysis, CD45$^+$ cells were gated, and the percentages of CD11c$^+$CD69$^+$ cells in the CD11c$^+$ cell population, CD3$^+$CD69$^+$ cells in the CD3$^+$ cell population, CD19$^+$CD69$^+$ cells in the CD19$^+$ cell population, and CD49b$^+$CD69$^+$ cells in the CD49b$^+$ cell population were calculated. Values represent means ± SD ($n = 4$ for each group).

[a] $p < 0.01$ versus group A.
[b] $p < 0.01$ versus group B.
[c] $p < 0.01$ versus group C.
[d] $p < 0.01$ versus group D.
[e] $p < 0.01$ versus group E.

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Table III. Comparison of CD40, CD80, and CD86 expression on decidual DCs (%)

Group	Mice	Stimulator	CD40	CD80	CD86
A	Jα18$^{+/+}$	PBS	19.9 ± 3.2	23.5 ± 2.5	18.2 ± 2.3
B	Jα18$^{-/-}$	PBS	19.7 ± 2.7	24.2 ± 3.2	18.5 ± 2.9
C	Jα18$^{+/+}$	LPS	47.4 ± 5.8^{a,b}	50.9 ± 4.2^{a,b}	61.7 ± 5.7^{a,b}
D	Jα18$^{-/-}$	LPS	21.9 ± 3.0^{c}	25.6 ± 2.9^{c}	18.7 ± 2.3^{c}

Pregnant Jα18$^{-/-}$ and Jα18$^{+/+}$ mice were i.p. injected with PBS or LPS at 50 μg/kg body weight. Decidual mononuclear cells were isolated and incubated with fluorescence-conjugated anti-CD45, anti-CD11c, anti-CD40, anti-CD80, and anti-CD86 mAbs and isotype controls. The CD45$^+$ cells were gated, and the percentages of decidual CD40$^+$ cells, CD80$^+$ cells, and CD86$^+$ cells in the CD11c$^+$ cell population were calculated. Data represent means ± SD of four samples in each group.

[a] $p < 0.01$ versus group A.
[b] $p < 0.01$ versus group B.
[c] $p < 0.01$ versus group C.

Discussion

The presence of iNKT cells at human decidua and their specific ligand CD1d at placenta villous and extravillous trophoblasts indicates that these cells may have an immunoregulatory role at the maternal/fetal interface (24, 25). Murine Vα14 NKT cells were found to accumulate in the decidua during pregnancy and induce abortion upon stimulation with their specific ligand, α-galactosylceramide, by perforin-dependent killing and production of IFN-γ and TNF-α (21, 26). Additionally, *Salmonella* LPS can stimulate iNKT cells dependent on TLR engagement of the APCs and recognition of a self glycolipid by the iNKT TCR (17). Furthermore, *Escherichia* LPS can drive iNKT cell production of IFN-γ via IL-12 and IL-18 production by activation of APCs independently of CD1d (27). These reports suggest that decidual iNKT cells may be activated indirectly by pathogen-related molecules such as bacterial LPS at the maternal/fetal interface, subsequently activating immune cell types, producing massive amounts of cytokines and playing a regulatory role in infection-associated abortion or preterm birth.

We previously reported that LPS treatment significantly increases the incidence of preterm delivery and fetal death and enhances expression of placental costimulatory molecule CD86 and activation molecule CD69 (18). In this study, we investigated the effect of iNKT cell deficiency on LPS-induced preterm birth and fetal demise. First, we showed that there was no difference in the gestation length of Jα18$^{+/+}$ mice and Jα18$^{-/-}$ mice. LPS given i.p. at 50 μg/kg body weight caused a ˃50% preterm birth rate in Jα18$^{+/+}$ mice. Therefore, it was concluded that the deficiency of iNKT cells decreased LPS-induced preterm birth and fetal demise. Alternatively, iNKT cells appear unnecessary for normal term delivery, since the gestation length was unchanged in Jα18 null mutant mice administered PBS.

LPS was also infused at 100 μg/kg body weight to determine whether iNKT cell depletion remained protective in the event of a stronger inflammatory challenge and was found to cause preterm delivery in 100% of Jα18$^{+/+}$ mice. Thus, deficiency of iNKT cells still reduced LPS-induced preterm birth and fetal demise with a high dose of LPS. Collectively, our results suggest that iNKT cells may have an important and essential role in inflammation-induced preterm birth. To our knowledge, this is the first study to address the role of iNKT cells in infection-associated preterm birth.

In the present study, we found that CD3$^+$CD49b$^+$ iNKT cells were absent or nearly undectable in Jα18$^{-/-}$ mice. In our investigation of the signaling mechanism by which iNKT cells mediate infection-associated preterm birth, we demonstrated that the expression of costimulatory molecules CD40, CD80, CD86, and CD69 in decidual DCs from iNKT cell-deficient Jα18$^{-/-}$ mice decreased significantly in comparison with those from Jα18$^{+/+}$ mice.

During pregnancy, the precise regulation of innate and adaptive immunity at the maternal/fetal interface is required to maintain the survival of the semiallogeneic embryo. At the same time, effective immunity must be maintained at the mucosal border to protect both mother and fetus from harmful pathogens. DCs are a heterogeneous population of cells that initiate and coordinate the innate and

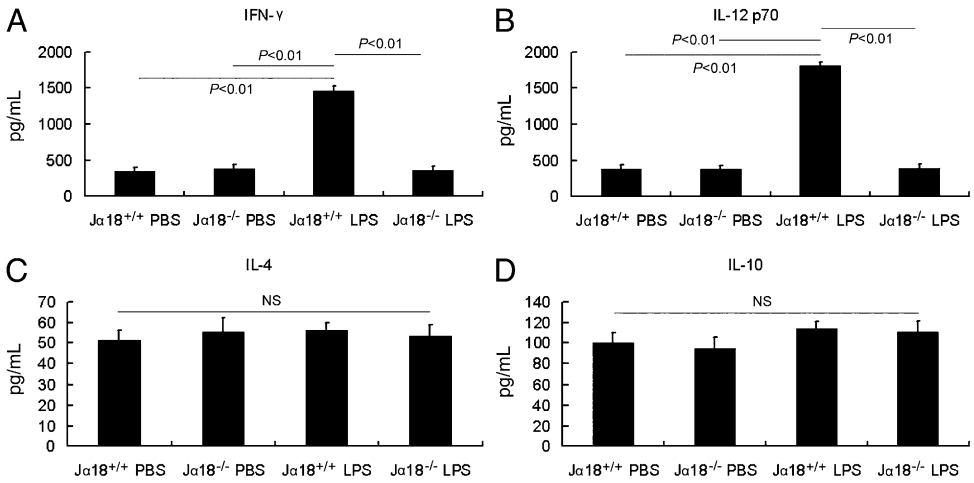

FIGURE 4. Analysis of cytokines in the culture supernatants of decidual mononuclear cells. Pregnant Jα18$^{-/-}$ or WT mice were injected i.p. with LPS to establish the preterm delivery model. Decidual mononuclear cells were isolated and cultured for 24 h. The culture supernatants were collected and stored at −80°C for cytokine assays. The concentrations of IFN-γ (**A**), IL-12p70 (**B**), IL-4 (**C**), and IL-10 (**D**) were determined by ELISA. Results are shown as means ± SD ($n = 4$ for each group).

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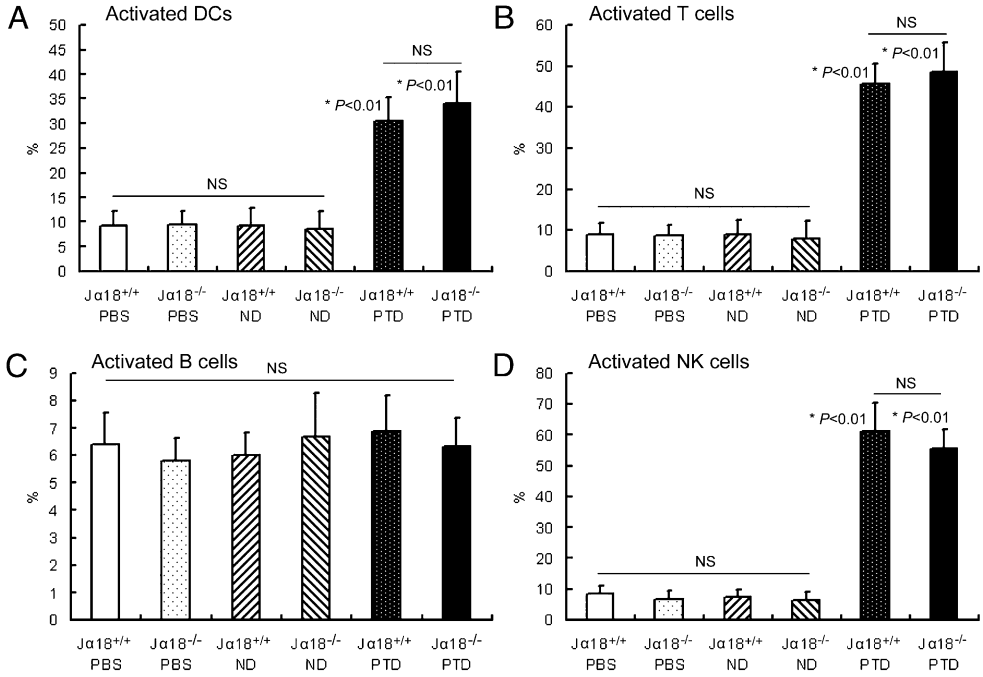

FIGURE 5. Comparison of the percentages of activated decidual DCs (**A**), T cells (**B**), B cells (**C**), and NK cells (**D**) between preterm delivery (PTD) and normal delivery (ND) mice. PBS-treated J$\alpha 18^{+/+}$ and J$\alpha 18^{-/-}$ mice were used as controls. Decidual tissues of LPS-treated PTD and ND mice were separated and examined respectively by flow cytometry ($n = 4$ for each group). *$p < 0.01$ compared with PBS-treated J$\alpha 18^{+/+}$ and J$\alpha 18^{-/-}$ mice as well as LPS-treated but ND J$\alpha 18^{+/+}$ and J$\alpha 18^{-/-}$ mice.

adaptive immune responses. Maternal DCs are scattered throughout the decidualized endometrium during all stages of pregnancy and appear to be important players in this fetal/maternal immune adjustment (28). Depletion of DCs has been shown to prevent blastocyst implantation and decidua formation, suggesting that uterine DCs are necessary for decidualization and affect the angiogenic response by inhibiting blood vessel maturation (29). Additionally, recent evidence points to a pivotal role of DCs in shaping the cytokine profile toward the establishment of a tolerogenic microenvironment at the maternal/fetal interface (30). Although DCs contribute to an immunosuppressive atmosphere over the course of gestation, they possess the capacity to contribute to proinflammatory responses upon pathogenic activation. Driven by pathogens and inflammatory signals, DCs undergo a complex maturation process, which not only leads to enhanced expression of costimulatory molecules and increased formation of stable MHC/peptide complexes but also to cytokine secretion modulating T cell activation and expansion, synthesis of chemokines and chemokine receptors, and regulation of T cell and DC trafficking (31). In our study, depletion of iNKT cells decreased the expression of costimulatory molecules CD40, CD80, and CD86 in decidual DCs and the number of activated decidual DCs, suggesting that iNKT cells have an impact on inflammation-induced decidual DC maturation and activation.

Productive activation of T cells occurs after concomitant engagement of TCR with Ag presented on DCs in association with MHC molecules and the delivery of costimulatory signals resulting from the interaction of CD80, CD86 with CD28, and CD40 with CD40L on the cell surface of T cells (32). Ag-specific T cell activation is a critical step in the rejection of transplanted allografts. As expected, our experiments demonstrated that decreased expression of decidual costimulatory molecules CD40, CD80, and CD86, as well as reduced activation of DCs, was consistent with downregulated decidual T cells and activated T cells. That the absence of iNKT cells decreased the numbers of T cells and activated T cells in decidua indicated that iNKT cells play a role in LPS-stimulated T cell expansion and activation.

Additionally, there were decreased numbers of NK cells and activated NK cells at the maternal/fetal interface of iNKT cell-deficient J$\alpha 18^{-/-}$ mice in comparison with J$\alpha 18^{+/+}$ mice, demonstrating that iNKT cells have an effect on infection-associated decidual NK cell expansion and activation. NK cells are lymphocytes characterized by high cytolytic potential against virus-infected and tumor-transformed cells. They play a fundamental role in the innate immune response through their ability to secrete cytokines and kill target cells without prior sensitization. Decidual NK cell cytolytic function is much reduced despite the presence of several activating receptors and the essential machinery required for lysis (33). Although decidual NK cell potential functions at the maternal/fetal interface are not yet clearly established, several hypotheses are being evaluated. It was proposed that NK cells in human decidua have a role in regulating trophoblast invasion by the production of IL-8 and IFN-inducible protein-10 chemokines (34). Furthermore, decidual NK cells secrete a potent array of angiogenic factors that induce vascular growth essential for the establishment of an adequate decidua (35, 36). Alternatively, we and others have proposed that those cells can become cytotoxic in nature in the presence of certain pathogens and ultimately lead to preterm birth (18, 37, 38). In our study, lack of iNKT cells caused downregulation of the NK cell population and activated NK cell proportion at the maternal/fetal interface, indicating that iNKT cells influence inflammation-induced decidual NK cell activation and cytotoxicity.

The conceptus is considered a semiallograft due to the presence of paternal HLA-C molecules. These alloantigens can be processed by maternal APCs, which present them to specific maternal $CD4^+$ T cells. After activation, the maternal $CD4^+$ T cells can become effector $CD4^+$ T cells, which are able to release various cytokines. $CD4^+$ helper T cells play important roles in an immune response to the Ag by modulating Ab production by B cells and the func-

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tion of cytotoxic T cells (39). T cell immunity is classified into Th1 and Th2 types by the cytokine profile. Th1-type cytokines that promote allograft rejection may compromise pregnancy, whereas the Th2 type cytokines that inhibit Th1 responses promote allograft tolerance and therefore may improve fetal survival (40). In our experiments, the levels of Th1 cytokines IFN-γ and IL-12p70 in the culture supernatants of decidual mononuclear cells from J$\alpha18^{-/-}$ mice were obviously decreased compared with those of J$\alpha18^{+/+}$ mice, whereas the concentrations of Th2 cytokines IL-4 and IL-10 were not markedly different. These results suggest that the absence of iNKT cells relieves the shift of infection-associated Th1/Th2 balance to a Th1 predominant state, thus promoting allograft tolerance.

The cause of preterm delivery is multifactorial (24), but chorioamnionitis and infection are major causes of preterm delivery <30 wk gestation in humans. In the present study, we did not find any significant difference in the percentages of peripheral blood DCs, T cells, B cells, and NK cells among PBS-injected or LPS-injected J$\alpha18^{+/+}$ mice and J$\alpha18^{-/-}$ mice. Our findings suggest that iNKT cells have a minor effect on systemic immune cells. To further explore the cause of preterm delivery, we compared the expression of CD69 on decidual DC, T cell, B cells, and NK cell populations among LPS-induced preterm delivered and LPS-treated but normally delivered J$\alpha18^{+/+}$ and J$\alpha18^{-/-}$ mice. Preterm delivered mice showed increased activation of DCs, T cells, and NK cells at the maternal/fetal interface in comparison with normally delivered mice, suggesting an essential role of those activated immune cells for infection-induced preterm delivery. Furthermore, the deficiency of iNKT cells decreased activation of decidual DCs, T cells, and NK cells, suggesting that iNKT cells may play a key role for activation of those immune cells in chorioamnionitis-induced preterm delivery. Our findings in mice altogether suggest that iNKT cells may play an important role in inflammation-induced preterm delivery, and in future studies we will investigate whether iNKT cells may also play such a role in humans.

Disclosures

The authors have no financial conflicts of interest.

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