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Effect of TLR3 and TLR7 activation in uterine NK cells from non-obese diabetic (NOD) mice☆

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Abstract

Toll-like receptor (TLR)–TLR cross talk is thought to be important in TLR signaling. Herein, we investigated the effect of specific TLR3 and TLR7 agonists, poly (I:C) and R837, individually and in combination, on uterine immune cell function and their subsequent effects on pregnancy outcome. Allogeneic pregnancies in the non-obese diabetic (NOD) mouse × C57BL/6 and wild-type BALB/c × C57BL/6 model were used. An additive increase in embryo resorption was observed after induction with both poly (I:C) and R837, and was associated with elevated numbers of both TNF-α- and IFN-γ-producing $CD45^+$ cells in the uterus. Further examination showed that while cytokine expression was detected in both $CD3^+$ cells and $CD49b^+$ cells in BALB/c mice, NOD mouse cells behaved differently. In NOD mice, elevated cytokine expression was attributed to $CD3^+$ T cells, with no response detected in the $CD49b^+$ NK cells. The additive effect of combined agonists was partially inhibited by the Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) inhibitor SP600125 and almost completely abrogated by the extracellular signal-regulated kinase (ERK) MAPK inhibitor PD98059. These results suggest that increased TLR3 and TLR7 signals are transmitted via Th1-type T cells, rather than NK cells, in NOD mice. Furthermore, the ERK MAPK pathway may be critical in TLR3 and TLR7 signaling.

Keywords: Abortion; Cell signaling; Immunodeficiency diseases; Placenta; uNK cell

1. Introduction

Innate recognition receptors are thought to recognize viral components and activate antiviral responses in immune and non-immune cells (Akira et al., 2006). Toll-like receptors (TLRs) are a family of innate pattern recognition receptors that can recognize virus-associated molecules and activate antiviral responses such as the expression of proinflammatory cytokines and type 1 interferons (Kawai and Akira, 2006). Among the TLR family members, TLR3 recognizes viral double-stranded (ds) RNA (Alexopoulou et al., 2001), and TLR7/8 recognizes viral single-stranded (ss) RNA (Heil et al., 2004).

Viral infection of macrophages initiates an innate antiviral immune response through TLRs (Steer et al.,

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2006) and mitogen-activated protein kinases (MAPKs) (Alexopoulou et al., 2001) resulting in the production of cytokines (Petro, 2005a, 2005b). One of these cytokines, IFN-β, induces an antiviral state and increases natural killer (NK) cell cytotoxicity (Biron et al., 1999). The cytokine IL-12 induces NK cell proliferation (Biron et al., 1999; Nguyen et al., 2002), NK cell production of IFN-γ (Chan et al., 1991) and macrophage nitric oxide (Pahan et al., 2001), thereby inducing potent antiviral processes. Recent research suggests that expression of IL-27 p28 by Theiler's virus-infected macrophages depends upon the activation of JNK-MAPKs by TLR3 and TLR7 (Hause et al., 2007).

A recent study using human peripheral blood mononuclear cells found that TLRs cross talk via complex mechanisms. Combinations of TLR agonists may or may not have advantages over single agonists in the induction of cytokine production (Ghosh et al., 2007). In another report, select TLR agonist combinations appeared to have a synergistic effect in triggering a T helper type 1 (Th1)-polarizing program in dendritic cells (DCs) (Napolitani et al., 2005). However, the effect of TLR–TLR cross talk in the mechanisms involved in pregnancy tolerance is still unclear.

Both T and NK cells are important immune cells in the pregnant uterus and are thought to play a critical role in the modulation of maternal tolerance to an allogeneic fetus (Saito et al., 2007, 2008). Previous research has shown that NK cell-deficient mouse models, such as TgE26 and NOD, were prone to spontaneous embryo loss and infertility. Restoring NK cell function improved pregnancy outcomes in TgE26 mice (Guimond et al., 1997, 1998) and NOD mice (Lin et al., 2009a; Wang et al., 2009). In contrast, depletion of NK cells increased embryo loss in NOD/SCID mice that retained a remnant of functional NK cells (Lin et al., 2005b, 2009b; Parsons et al., 2006). In the present study, we aimed to identify the presence of cross talk between TLR3 and TLR7. Furthermore, we used an NOD model, characterized by a deficiency of NK cells (Kataoka et al., 1983) and reduced fertility (Burke et al., 2007; Wang et al., 2009), to determine if the specific agonists of TLR3 and TLR7 synergistically influence the functional status of uterine T cells and NK cells.

2. Materials and methods

2.1. Reagents

TLR7 agonist imiquimod (1-(2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine (R-837; S-26308); InvivoGen, San Diego, CA) was dissolved in dimethyl sulfoxide (DMSO; sterile cell culture grade, Sigma–Aldrich, St. Louis, MO) at a concentration of 12 mM and stored in aliquots at −20 °C. Polyinosinic–polycytidylic acid [poly (I:C)] is a synthetic, double-stranded RNA and is a known ligand of TLR3. Poly (I:C) (γ ray-irradiated, Sigma–Aldrich) was dissolved in phosphate-buffered solution (PBS) and was adjusted to the desired concentration as necessary.

2.2. Pregnant model and embryo loss

Ten to 12-week-old female BALB/c and NOD mice and male C57BL/6 inbred mice (Experimental Animal Center of Zhongshan University, Guangzhou, China) were housed under pathogen-free conditions. Female BALB/c and NOD mice were co-caged with C57BL/6 males, generating naturally established allogeneic pregnant BALB/c × C57BL/6 and NOD × C57BL/6 models. The day on which a vaginal plug was detected was designated day 0.5 of gestation. All animal procedures followed the animal care guidelines of Jinan University, and all related data were approved for publication by the University's Institutional Review Board.

Pregnant mice were intraperitoneally (i.p.) injected a total of three times with poly (I:C) (200 ng/ml, in a volume of 200 μl, with R837 (20 ng/ml; 200 μl), with a combination of poly (I:C) and R837 (40 ng poly (I:C) in 100 μl PBS and 4 ng R837 in 100 μl DMSO), or with an equal volume of PBS or DMSO as a solvent control, at gestational days 2.5, 4.5, and 6.5, respectively. Embryo-depleted placentas and associated decidual tissues including the decidua basalis, were harvested at day 12.5 using previously described techniques (Lin et al., 2006).

Abortion (resorption) sites were identified by their small size, accompanied by a necrotic, hemorrhagic appearance relative to normal embryos and placentas. The percentage of resorptions was calculated as the ratio of resorption sites and total implantation sites (resorption plus normal implantation sites), as described previously (Lin et al., 2004; Zenclussen et al., 2006).

2.3. TLR-agonists on intracellular cytokine production in uterine $CD45^+$ cells

CD45, a well-characterized common leukocyte antigen, is extensively expressed on leukocytes, and is used here as a surface marker of leukocytes (Lin et al., 2006). The female mice, previously injected with poly (I:C), R837, poly (I:C) plus R837, solvent control PBS or DMSO at gestational days 2.5, 4.5, and 6.5, were killed at day 12.5 to collect placentas and decidua basales.

The pooled placentas from each mouse were carefully cut into small pieces (<1 mm^3) with ocular scissors. The pieces then were collected in Hank's buffered salt solution (NaCl, 8 g; KCl, 0.4 g; $CaCl_2$, 0.14 g; $MgSO_4$, 0.2 g; Na_2HPO_4, 0.06 g; $NaHCO_3$, 0.35 g; and glucose, 1 g in 1 l distilled water) and filtered through a 50 μm pore size nylon mesh (Guangzhou Reagent Company, Guangzhou, China) to obtain a mononuclear cell suspension. Lymphocytes were purified by centrifugation on a ficoll-hypaque density gradient and incubated with red cell lysis buffer (eBioscience, San Diego, CA) at 37 °C for 10 min to eliminate red blood cells. The cells were incubated with PE-conjugated anti-mouse CD45, washed once with PBS, resuspended and incubated in permeabilization buffer (EBioscience Inc.) for 1 h, and then stained with an FITC- or Alexa Fluor 488-conjugated anti-cytokine antibody for TNF-α, IFN-γ, IL-4 and IL-10 (all from EBioscience Inc.), respectively. For the indirect staining of transforming growth factor (TGF)-β1, cells were incubated with mouse anti-human/mouse TGF-β1 (US Biological, Swampscott, MA) and stained with an FITC-conjugated rat anti-mouse immunoglobulin (Caltag Laboratories). Finally, the stained cells were assayed on an FACS Calibur flow cytometer (BD Biosciences). Isotype controls were established by staining with PE, FITC or Alexa Fluor 488-conjugated isotype antibodies to exclude false positive cells (Lin et al., 2006, 2009b).

2.4. The in vitro effect of TLR3 and TLR7 agonists on cytokine production in uterine $CD3^+$ or $CD49b^+$ cells

Under sterile conditions, MACS was used to purify placental $CD3^+$ and $CD49b^+$ cells from non-stimulated BALB/c × C57BL/6 and NOD × C57BL/6 mice on gestational day 12.5. In brief, single cells were incubated with microbead-conjugated anti-mouse CD3 or anti-mouse CD49b, and purified through mini-size MACS separation columns (all from Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions. The purity and viability of the purified cells routinely exceeded 97% and 95%, respectively, as determined by flow cytometry and propidium iodide staining (Invitrogen, Eugene, OR) (Fischer et al., 2002; Lin et al., 2009b).

Purified $CD3^+$ and $CD49b^+$ cells were seeded onto 24 well plates at a density of 10^6 cells/ml in RPMI1640 medium supplemented with fetal bovine serum (Sigma–Aldrich). Poly (I:C) (final concentration 50 μg/ml), R837 (final concentration 10 μg/ml), or a combination of both was added into the culture medium. After 6 days of cultivation, the cells were harvested, and the percentages of $CD3^+$ and $CD49b^+$ cells positive for intracellular TNF-α were detected by flow cytometry and compared with solvent control groups. Brefeldin A (10 μg/ml; Sigma–Aldrich) was added to each well 6 h before harvesting to allow for the accumulation of intracellular cytokines. Isotype controls were also established, as described above.

2.5. Standard ^{51}Cr release assay

Based on naturally established BALB/c × C57BL/6 and NOD × C57BL/6 pregnant mouse models, $CD49b^+$ NK cells were purified under sterile condition from spleens or pooled placentas and decidua basales at day 12.5 of gestation by MACS. Cells were cultured in complete RPMI1640 medium containing 10% fetal bovine serum in the presence of poly (I:C) (final concentration, 200 ng/ml), R837 (100 ng/ml), poly (I:C) and R837 (final concentrations, 200 and 100 ng/ml, respectively) or an equal volume of PBS or DMSO. After 72 h cultivation, the cells were harvested and washed twice with PBS, resuspended in complete medium and titrated two-fold on 96 well plates. ^{51}Cr-labeled (PerkinElmer, Boston, MA) YAC-1 target cells (5×10^3 cells/well; from American Type Culture Collection, Rockville, MD) were subsequently added to the wells. Each assay was performed in triplicate. After 4 h of incubation, the percentage of specific lysis was measured by a γ counter and calculated by the following formula: percentage specific lysis = (experimental c.p.m. − spontaneous release c.p.m.)/(total c.p.m. − spontaneous release c.p.m.) × 100. Total c.p.m. (counts per minute) was determined from the wells receiving ^{51}Cr-labeled YAC-1 target cells and 2.5% Triton X-100 (Sangon Inc., Shanghai, China) (Caraux et al., 2006).

2.6. The effects of selected inhibitors on TLR3 and TLR7 signaling

Under sterile conditions, placental $CD3^+$ cells from NOD mice on gestational day 12.5 were seeded onto 24 well plates at a density of 10^6 cells/ml in RPMI1640 medium supplemented with fetal bovine serum (Sigma–Aldrich) and cultivated for 24 h. Cells were treated with SP600125, an inhibitor of c-Jun N terminal kinase (JNK) MAPK, PD98059, an inhibitor of extracellular signal-regulated kinase (ERK) MAPK (both from Sigma–Aldrich; final concentration: 100 μmol/l) or the same volume of DMSO and cultivated for 24 h (Biosvieux-Ulrich et al., 2005). Cells were then treated with poly (I:C) (50 μg/ml), R837 (10 μg/ml) or a combination of both and cultured for 72 h. Brefeldin

A (10 μg/ml; Sigma–Aldrich) was added to each well 6 h before harvesting to allow for the accumulation of intracellular cytokines. Cells were harvested and stained with FITC-conjugated anti-mouse CD3 (eBioscience), permeabilized and intracellularly stained with PE-conjugated anti-TNF-α. Cells were then washed and analyzed by flow cytometry. Isotype controls were also established, as described above.

2.7. Statistical analysis

Embryo loss data were compared by χ^2 tests. Flow cytometric data were analyzed using Quad statistics (Lin et al., 2005a, 2006). Cell subset percentage data were compared by Student's *t* test, and the results were expressed as means ± SEM (standard error of the mean) (Lin et al., 2005a, 2006).

3. Results

3.1. Effect of combined TLR3 and TLR7 agonists on embryo loss

An additive increase in the embryo resorption rate in NOD × C57BL/6 mice was observed after the injection of both poly (I:C) and R837 (Fig. 1). The resorption rates in the single agonist injection groups were 38.3 ± 4.5% for the poly (I:C) group and 35.8 ± 4.3% for the R837 group. Both were significantly higher than the corresponding solvent control groups (17.5 ± 4.2% for PBS group and 16.3 ± 3.3% for DMSO group; $P < 0.01$ for both). In comparison, the resorption rate in the poly (I:C) and R837 combination group was increased to 64.3 ± 6.7%. This rate is significantly higher than that in either the poly (I:C) group or the R837 group ($P < 0.01$ for both).

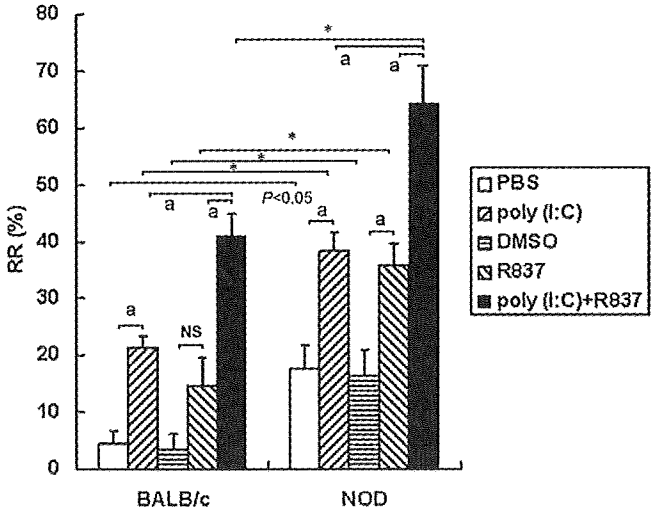

Fig. 1. The additive effect of poly (I:C) and R837 on the induction of embryo resorption. RR, embryo resorption rate. $n = 6$ for each group. *P* values are indicated where necessary. NS, not significant.
[a] $P < 0.01$ between different treatment within the same group of mice.
[*] $P < 0.01$ between similar treatments in different groups of mice.

A similar trend was observed in BALB/c × C57BL/6 mice (Fig. 1). The resorption rates in the poly (I:C) and the R837 groups were 21.4 ± 2.9% and 14.6 ± 5.0%, respectively. Resorption rates markedly increased when both poly (I:C) and R837 were injected (41.1 ± 3.1%, $P < 0.01$ for both). These results suggest that these TLR3 and TLR7 agonists additively increase the resorption rate in both NOD and BALB/c mice.

In addition, the resorption rates in NOD mice were considerably higher relative to the corresponding groups in BALB/c mice (Fig. 1) ($P < 0.05$ for PBS groups and $P < 0.01$ for DMSO, poly (I:C), R837, and poly (I:C) plus R837 groups).

3.2. The in vivo effect of combined TLR3 and TLR7 agonists on intracellular cytokines in the uterine $CD45^+$ cell population

The percentages of cells positive for TNF-α, IFN-γ, IL-4, TGF-β and/or IL-10 in uterine $CD45^+$ cell population are shown in Fig. 2. In general, the TNF-α^+ and IFN-γ^+ percentages in $CD45^+$ cells were markedly increased upon induction with poly (I:C) or R837. Percentages were further increased upon treatment with both poly (I:C) and R837 (Fig. 2A and B). In contrast, no such trends were observed in the percentages of IL-4^+, TGF-β^+, or IL-10^+ cells. However, the percentages in some BALB/c groups were significantly higher than the corresponding NOD groups. These results suggest that, in some cases, the functional status of uterine $CD45^+$ cells derived from NOD mice may be different from those derived from BALB/c mice (Fig. 2C–E).

3.3. The in vitro effect of combined TLR3 and TLR7 agonists on the intracellular production of cytokines in uterine $CD3^+$ and $CD49b^+$ cell populations

In BALB/c mice, the percentage of $CD3^+$ and $CD49b^+$ cells positive for TNF-α increased significantly upon individual stimulation with poly (I:C) or R837 and was increased even further in response to the combined stimulation of these TLR agonists ($P < 0.01$ for all; Fig. 3A1–A6, B1–B6, E).

In NOD mice, the percentage of $CD3^+$ cells positive for TNF-α was significantly increased upon stimulation with poly (I:C) or R837 and this percentage was further increased by the combination of poly (I:C) and R837

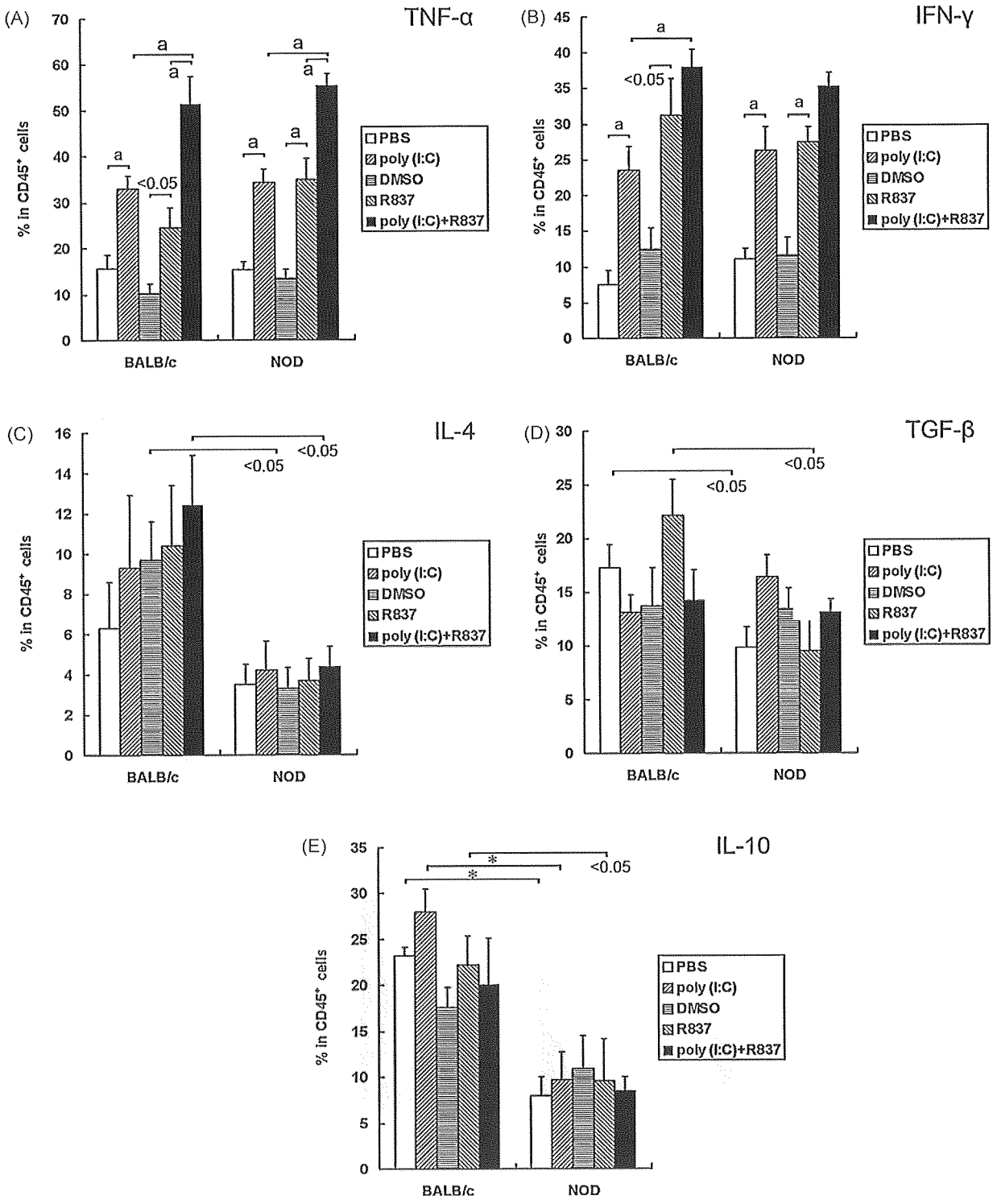

Fig. 2. The additive effect of combined *in vivo* poly (I:C) and R837 stimulation on the increase of intracellular TNF-α and IFN-γ production in placental leukocytes. The percentage positive for the indicated cytokines in the $CD45^+$ cell population was compared. (A) TNF-α; (B) IFN-γ; (C) IL-4; (D) TGF-β; and (E) IL-10. $n = 4$ for each group. P values are indicated where necessary.

[a] $P < 0.01$ between different treatments within the same group of mice.

* $P < 0.01$ between similar treatments in different groups of mice.

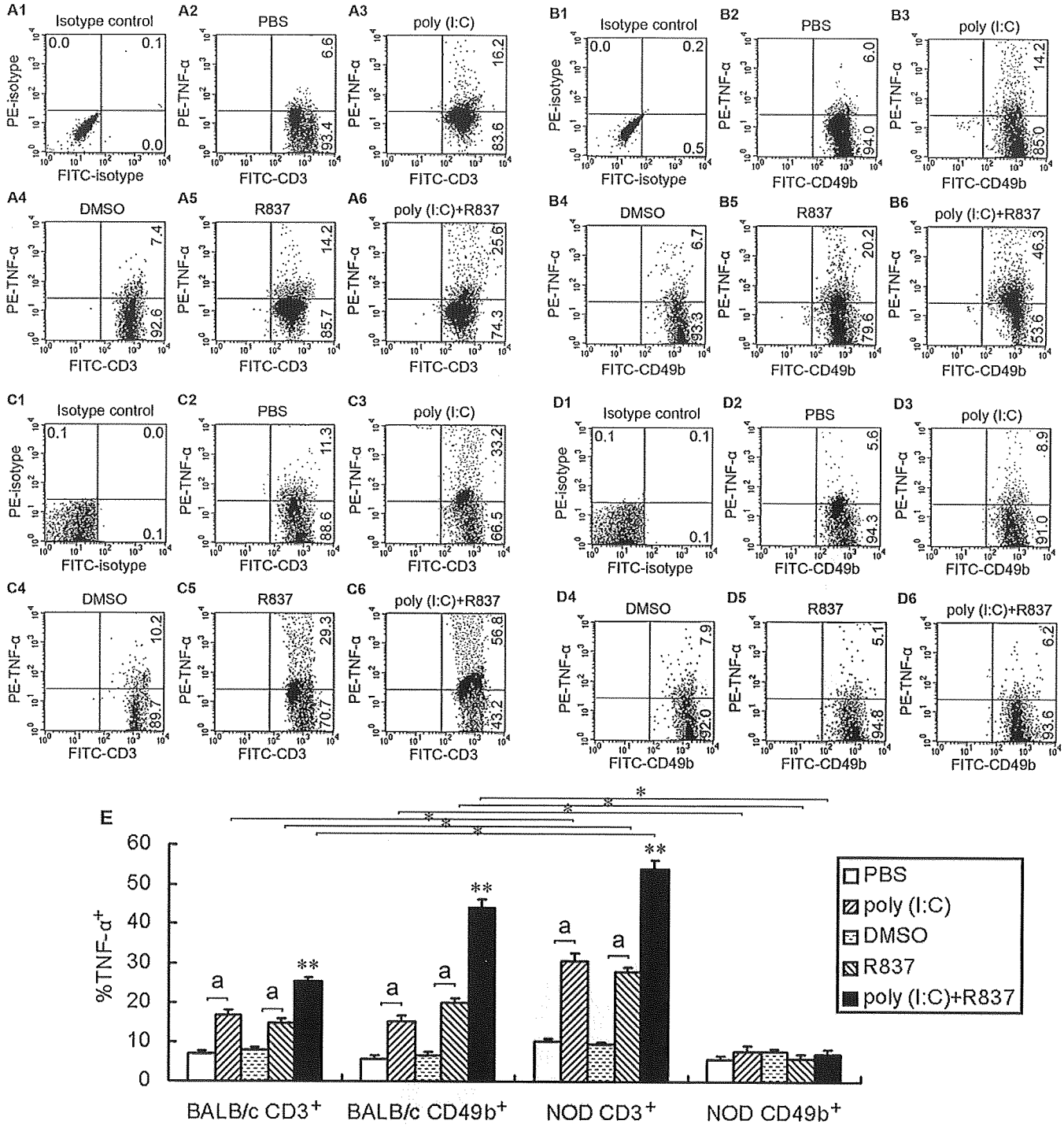

Fig. 3. Differential increase of TNF-α production in $CD3^+$ and $CD49b^+$ cells upon the stimulation of poly (I:C), R837 or their combination. Data are from placentas and decidua basales (PL) of BALB/c × C57BL/6 and NOD × C57BL/6 mice. (A1–A6) BALB/c, $CD3^+$ cells; (B1–B6) BALB/c, $CD49b^+$ cells; (C1–C6) NOD, $CD3^+$ cells, and (D1–D6) NOD, $CD49b^+$ cells. (E) Summary of flow cytometric data. $n = 4$ for each group. P values are indicated where necessary.

[a] $P < 0.01$ between different treatments within the same group of mice.

* $P < 0.01$ between similar treatments in different groups of mice.

** $P < 0.01$ versus the other four kinds of treatment [PBS, poly (I:C), DMSO and R837] within the same group of mice.

($P<0.01$ for all; Fig. 3C1–C6, E). However, the NOD TNF-α^+CD49b$^+$ cell percentage was not increased when stimulated by either poly (I:C), or R837, or a combination of both ($P>0.05$ for all; Fig. 3D1–D6, E).

3.4. NK cell cytotoxicity as determined by standard ^{51}Cr release assay

As shown in Fig. 4A and B, the cytotoxicity of splenic NK cells derived from BALB/c mice was significantly increased by either agonist alone and was further increased with both agonists. In contrast there was lower cytotoxicity in NOD splenic NK cells. This cytotoxicity was slightly increased upon single agonist stimulation and increased further upon combined stimulation. In uterine NK (uNK) cells from BALB/c mice, the cytotoxicity was increased significantly by either single agonist and was increased further upon induction with both. However, the level of cytotoxicity was significantly lower than that observed in cells derived from BALB/c spleens. Uterine NK cells isolated from NOD mice had a much lower cytotoxicity. This cytotoxicity was not increased in response to the stimulation of either single or combined agonists (Fig. 4C and D).

3.5. The effects of SP600125 and PD98059 on TLR3 and TLR7 signaling

As shown in Fig. 5, the increase in the proportion of TNF-α^+CD3$^+$ cells by poly (I:C), R837 or a combination of both poly (I:C) and R837 was partially abrogated by SP600125. This increase was abrogated to a great extent by PD98059. This trend was observed in both BALB/c and NOD mice.

In BALB/c mice without either antagonist, the percentages of TNF-α^+CD3$^+$ cells were $17.9\pm1.0\%$, $16.4\pm1.1\%$ and $25.1\pm1.4\%$ in the poly (I:C) group, the R837 group, and the combination stimulation group, respectively. Addition of SP600125 to the cells decreased the percentages to $11.6\pm0.8\%$ ($P<0.01$), $11.5\pm0.9\%$ ($P<0.05$) and $19.4\pm1.5\%$ ($P<0.05$), respectively. Addition of PD98059 decreased the percentages further to $7.6\pm0.5\%$, $8.3\pm0.5\%$ and $10.8\pm1.0\%$, respectively. In each case, the percentage of TNF-α^+CD3$^+$ cells was significantly lower than that of cells without any antagonist ($P<0.01$ for all) (Fig. 5A1–A4, B1–B4, C1–C4, G).

In NOD mice, the percentages of TNF-α^+CD3$^+$ cells were $22.5\pm1.4\%$, $18.0\pm1.3\%$, and $36.2\pm1.9\%$ in the poly (I:C) group, the R837 group and the combination

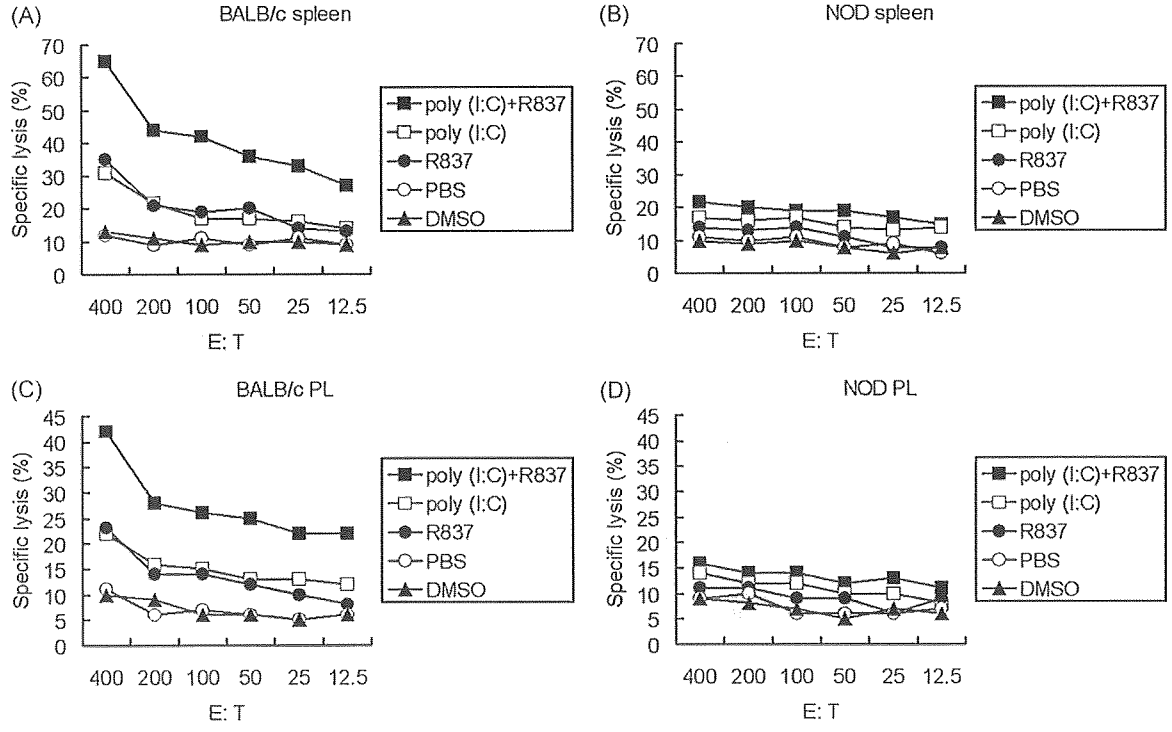

Fig. 4. CD49b$^+$ NK cell cytotoxicity detected by ^{51}Cr release assay. (A) BALB/c spleen. NK cell cytotoxicity was increased significantly by either agonist alone and was increased further by combined stimulation with both agonists. (B) NOD spleen. There was a lower, but detectable, cytotoxicity, which was slightly increased upon single agonist stimulation and increased further upon combined stimulation. (C) BALB/c placenta and decidua basalis. NK cell cytotoxicity was increased significantly by either agonist alone and was increased further by both agonists. However, all levels were significantly lower than those observed in the BALB/c spleen group. (D) NOD placenta and decidua basalis. There was a lower, but detectable, cytotoxicity, which was not increased in response to stimulation by either single or combined agonists.

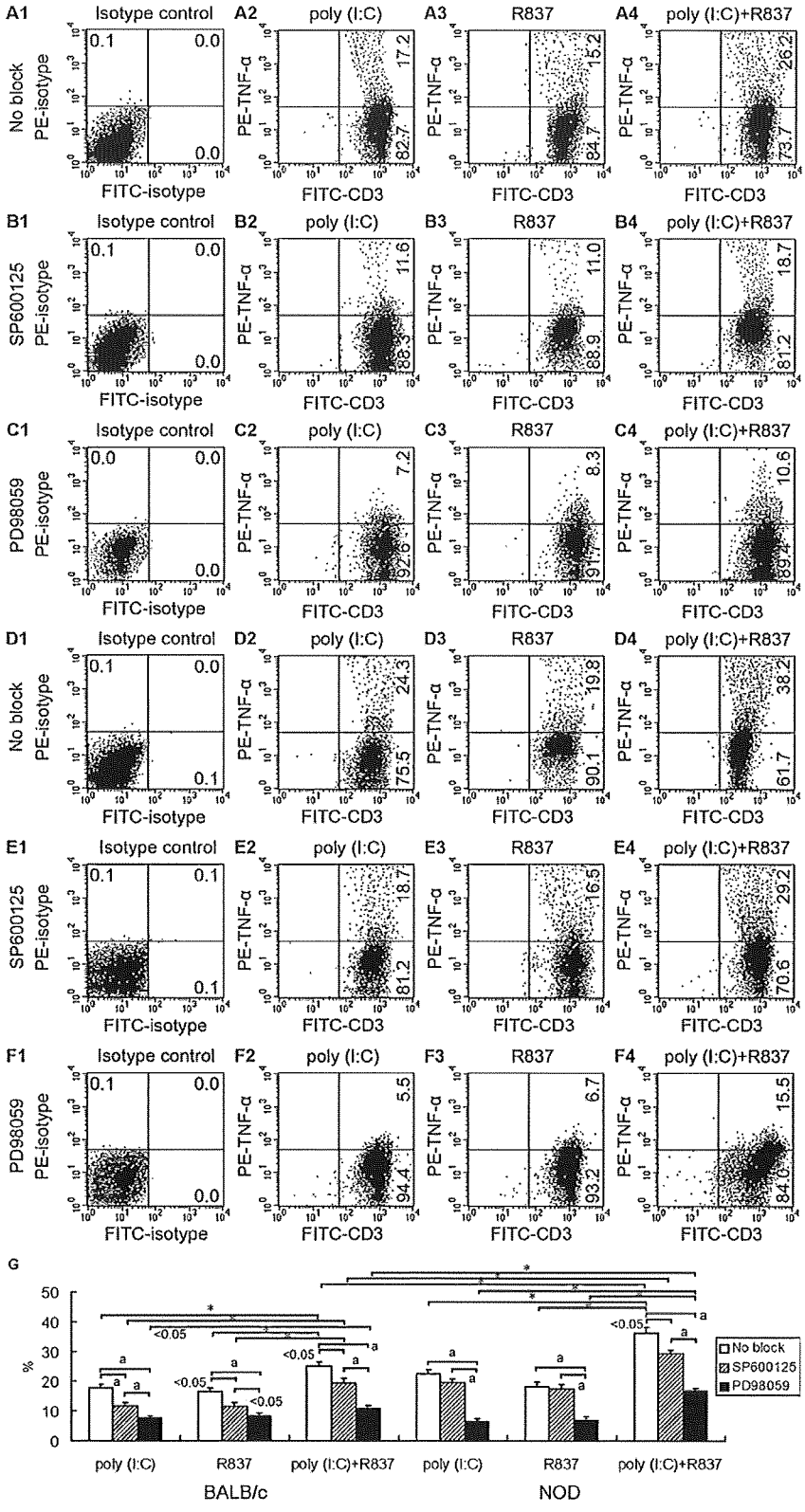

Fig. 5. The effect of SP600125 and PD98059 on the poly (I:C)- and R837-induced increase of TNF-α production. $CD3^+$ T cells were purified by MACS from gestational day 12.5 placentas and decidua basales, cultured and stimulated with poly (I:C), R837 or both, in the presence or absence of SP600125 and PD98059. (A1–A4, B1–B4, C1–C4) BALB/c mice, and (D1–D4, E1–E4, F1–F4) NOD mice. (G) Summary of flow cytometric data. $n = 4$ for each group. "%" indicates the mean of the percentages positive for TNF-α in the $CD3^+$ population. "No block" indicates the DMSO solvent control group. P values are indicated where necessary.

[a] $P < 0.01$ between different treatments within the same group of mice.

[*] $P < 0.01$ between similar treatments in different groups of mice.

stimulation group, respectively. Addition of SP600125 decreased these percentages to $19.5 \pm 1.1\%$ ($P > 0.05$), $17.5 \pm 1.2\%$ ($P > 0.05$) and $29.3 \pm 1.2\%$ ($P < 0.05$), respectively. Addition of PD98059 decreased these percentages to $6.5 \pm 0.9\%$, $7.0 \pm 0.8\%$, and $16.5 \pm 1.0\%$, respectively. These decreases were significantly lower than those of cells without any antagonist ($P < 0.01$ for all) (Fig. 5D1–D4, E1–E4, F1–F4, G).

Although the percentage increase of TNF-α^+CD3$^+$ cells induced by both poly (I:C) and R837 was significantly weakened by either SP600125 or PD98059, these percentage levels were still significantly higher than those of cells induced by either poly (I:C) or R837 alone. This suggest that there is an additive effect of having both poly (I:C) and R837 (Fig. 5G).

4. Discussion

Recent data have shown that components of the immunoregulation system, including CD4$^+$CD25$^+$ regulatory T (Treg) cells, Th3 cells, Tr1 cells, regulatory NK cells and the tryptophan-catabolizing enzyme indolamine-2,3-deoxygenase, play critical roles in the maintenance of pregnancy. Both Treg cells and regulatory NK cells may inhibit maternal T or NK cells from attacking a fetus (Saito et al., 2007). In addition, a balance between cytotoxic NK cells and regulatory NK cells must exist for viable human pregnancies (Saito et al., 2008).

To investigate the functions of T and NK cells and their activation by TLR agonists, we used NOD mice, which are deficient in NK cells and are prone to insulin-dependent diabetes mellitus (IDDM). Using flow cytometry, fewer peripheral NK cells were detected in NOD mice relative to normal C57BL/6 and other strains of mice. This decrease in peripheral NK cells was associated with an increase of bone marrow NK cells, suggesting that a defect in NK cell export from bone marrow to peripheral tissues may be responsible (Poulton et al., 2001).

In the present study, we investigated the effect of TLR3 and TLR7 agonists on cytokine production in CD3$^+$ T cells and CD49b$^+$ NK cells, which represent major leukocyte populations in the pregnant mouse uteri. To some extent, cytokine production in other uterine CD45$^+$ cells may reflect the trends of cytokine production in CD3$^+$ T cells plus CD49b$^+$ NK cells. A synergistic effect was not observed in response to combined poly (I:C) and R837 stimulation. However, poly (I:C) and R837 additively increased TNF-α and IFN-γ production in CD45$^+$ uterine cells, resulting in an additive increase in embryo loss in both wild-type and NOD allogeneic pregnant models.

Cytokine-producing CD45$^+$ cells were further investigated, with CD3 serving as a pan-marker for mature T cells and CD49b as a pan-marker for uNK cells (Arase et al., 2001). Since CD49b is also expressed by a small fraction of other cells, including B cells and monocytes, it is not an ideal pan-NK cell marker (Arase et al., 2001). However, as a better marker does not exist for NK cells, CD49b is a commonly accepted pan-NK cell marker in murine models (Arase et al., 2001; Wang et al., 2009). Fortunately, as most leukocytes in the decidua comprise NK cells, CD3$^+$ T cells and other cells, while B cells are virtually absent (von Rango et al., 2001), the possibility of B cell contamination is very small.

Stimulation of TLR agonists induced Th1-type cytokine production in both T cells and NK cells isolated from BALB/c mice. These results suggest that both T cells and NK cells may play important roles in the prevention of viral infection, in addition to their roles in the modulation of pregnancy tolerance in wild-type mice. In contrast, only CD3$^+$ T cells seem to play a role in the prevention of viral infection in NOD mice.

We have previously reported that NK cells can be classified into conventional NK cells and regulatory NK cells (Lin et al., 2008). We have also reported that NOD/SCID mice have only regulatory NK cells (Lin et al., 2005b). Regulatory NK cells may be the predominant NK cell population in NOD mice. Previous studies have shown that the cytotoxicity of peripheral blood NK cells from NOD mice (Kataoka et al., 1983; Carnaud et al., 2001; Poulton et al., 2001) and certain IDDM patients (Nair et al., 1986; Negishi et al., 1986) against NK sensitive tumor cell targets (e.g., K562 and YAC-1) was lower than that observed when using NK cells from normal, healthy mice or humans. Splenic NK cells from NOD mice displayed significantly weaker cytotoxicity against YAC-1 target cells (Ogasawara et al., 2003). In the present study, similar results were observed in NK cells derived from both the spleens and pregnant uteri of NOD mice. These results suggest that there are functional differences between the NK cells of normal mice and those of NOD mice, and similarly there may be differences between NK cells from normal, healthy people and those from some IDDM patients.

Notably in the present study, NOD CD3$^+$ T cells appeared to be more sensitive to stimulation by selected TLR agonists than CD3$^+$ T cells obtained from BALB/c mice. This may be harmful to pregnancy outcome in NOD mice and may be helpful in explaining why these mice are more sensitive to poly (I:C)- and R837-induced embryo resorption.

Although T cell- and NK cell-deficient NOD/SCID mice display normal fertility in general, NK cell-deficient NOD mice display a markedly increased percentage of embryo loss. Additionally, NOD NK cells were poorly activated by TLR ligands. We conclude, therefore, that T cells are responsible for promoting abortion (Lin et al., 2005b). It would be interesting to see if RAG 1 and RAG 2 knockout mice (Su et al., 2006; Ha et al., 2006) and some similar strains, which suffer from a deficiency of T cells but not NK cells, are less prone to abortion. However, as Treg cells may be critical in the success of allogeneic pregnancy, fertility of some T cell-deficient mice may be damaged if the mice also suffer from a deficiency of Treg cells (Aluvihare et al., 2004).

MAPKs play critical roles in many physiological processes, including cell growth, cell differentiation, apoptosis and the immune response, by mediating the production of cellular signals by extracellular stresses (Lien et al., 2006; Lin et al., 2009b). In this study, PD98059 almost completely blocked the poly (I:C)- and R837-induced increase in TNF-α production in $CD3^+$ cells derived from NOD mice. In contrast, SP600125 only slightly inhibited the increase in TNF-α production by a combination of poly (I:C) and R837. These results indicate that the ERK MAPK pathway may play a critical role in TLR3 and TLR7 signaling. Furthermore, these results suggest that the ERK MAPK pathway may be more critical in the transmission of increased TLR3 and TLR7 signals.

It will be necessary to clarify which cells express TLR3 and TLR7 intracellularly amongst T cells, NK cells and other types of cells. We must also clarify whether MACS-purified T cells and NK cells express TLR3 and TLR7 and respond to TLR agonists directly, or instead, whether they rely on antigen presenting cells (APCs) such as DCs, which act as professional APCs and respond to the agonists indirectly. Although it is unlikely that there are any APCs remaining among the MACS-purified T cells and NK cells used in this study, further research is required to confirm this assumption.

A recent report confirmed that TLRs1–13 (including TLR3 and TLR7) were expressed by purified (purity greater than 99%) $CD4^+$ and $CD8^+$ T cells from C57BL/6 and BALB/c mice (Salem et al., 2009). Human uNK cells are known to express several TLRs, including TLR2–4 (Eriksson et al., 2006). Other researchers have found that TLR7 is expressed by murine $CD3^-CD49b^+$ NK cells (Sawaki et al., 2007). In summary, these results support the conclusion that TLR3 and TLR7 are expressed in both T cells and NK cells in humans as well as mice.

Some TLR family members, such as TLR1, TLR2, TLR4–6 and TLR10, are expressed on the surface of cells, whereas other TLRs are predominantly expressed intracellularly, e.g., TLR3, TLR7–9 (Sen and Sarkar, 2005). In the present study, the synthetic ligands used are presumably internalized and interact with TLR3 and TLR7 in intracellular compartments. The TLR8 ligand R848 (imidazoquinoline) and the TLR9 ligand CpG may function in the same way (Lin et al., 2009b; Torii et al., 2008; Crompton et al., 2009).

In the present study, TLR3 and TLR7 appeared to promote the production of Th1-type cytokines, including INF-γ and TNF-α. However, TLR3 and TLR7 ligands failed to induce a Th2-type response, as Th2-type cytokine levels did not increase significantly upon stimulation with poly (I:C) alone, R837 alone, or their combination. It is known that thymic stromal lymphopoietin (TSLP) can induce a Th2-type response in both pregnant uteri and extra-uterine tissues, as distinct from the poly (I:C)-induced response (Zhou et al., 2005; Lin et al., 2008, 2009c). Thus, it is possible that TSLP participates in the modulation of allogeneic pregnancy tolerance, whereas TLR3 and TLR7 participate in the induction of Th1-type cytokine production and other antiviral responses.

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Toll-like receptor signaling in uterine natural killer cells—role in embryonic loss

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ABSTRACT

Embryonic development is a complex process that is regulated by many cell types and signaling pathways. This review focuses on the role of NK cells and regulatory T-cells (Treg cells) in embryonic loss. Approximately 70% of uterine leukocytes until the time of mid-gestation are found to be $CD16^{-}CD56^{bright}$ NK cells. This subset of NK cells, along with Treg cells, has been shown to regulate fetal development. We recently found a population of NK cells in the pregnant mouse uterus with a unique $CD3^{-}CD49b^{+}CD25^{+}Foxp3^{+}$ phenotype. This review summarizes the studies indicating critical roles for expression of IL-10 by $CD3^{-}CD49b^{+}CD25^{+}Foxp3^{+}$ cells and CXCR4 expression on $CD16^{-}CD56^{bright}$ NK cells in preventing embryonic loss. In addition, the roles of toll-like receptors (TLRs) and CXCR4 in NK cell migration and functional modulation are discussed.

1. Uterine NK (uNK) cells and toll-like receptors (TLRs)

1.1. Regulatory NK cells

In recent years, a novel concept has emerged that the actual role of natural killer (NK) cells is not confined to the destruction of virus-infected cells or tumors. Indeed, different NK subsets exist that display major functional differences in their cytolytic activity, cytokine production and homing capabilities (Higuma-Myojo et al., 2005). In particular, human $CD16^{-}CD56^{bright}$ NK cells, predominant in the pregnant uterus and lymph nodes, have little cytolytic activity but release high levels of cytokines. In contrast, $CD16^{+}CD56^{dim}$ NK cells, which predominate in peripheral blood and inflamed tissues, display lower cytokine production but potent cytotoxicity. The latter is characterized by granule polarization and exocytosis of various proteins including perforin and granzymes, which mediate target cell killing. The recruitment of $CD16^{+}CD56^{dim}$ NK cells into inflamed peripheral tissues is orchestrated by various chemokines, including the newly identified chemerin (Moretta et al., 2008). At these sites, upon interaction with myeloid dendritic cells (DCs) and engagement of different triggering receptors, NK cells become activated and up-regulate their cytokine production and cytotoxicity. Importantly, during this interaction, NK cells also mediate the 'editing' of DCs undergoing maturation. This process appears to play a crucial role in shaping both innate and adaptive immune responses (Moretta et al., 2008).

Under physiological conditions, more than 70% of uterine lymphocytes are NK cells until mid-gestation. These cells are characterized by the $CD16^{-}CD56^{bright}$ phenotype in humans and the $CD49b^{+}$ phenotype in mice (Nishikawa et al., 1991; Moffett-King, 2002). Uterine NK cells, especially those that accumulate in the decidual basalis, are believed to be involved in mechanisms that influence reproduction. More specifically, they are thought to medi-

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ate a mucosal immunological balancing function that prevents over-invasion of trophoblast cells into the maternal blood supply while still allowing a degree of placental access. A compromise is reached and both maternal and fetal gene systems, including genes expressed by NK cells and NK cell receptor genes expressed by target cells, might affect this compromise (Moffett-King, 2002; Lin et al., 2009b).

Both T and NK cells are present in the pregnant uterus and are thought to play a critical role in the modulation of maternal tolerance to an allogeneic fetus (Saito et al., 2007, 2008). Previous research has shown that NK cell-deficient mouse models, such as TgE26 and NOD, are prone to spontaneous embryo loss and infertility. Restoring NK cell function improves pregnancy outcomes in TgE26 mice (Guimond et al., 1998) and NOD mice (Wang et al., 2009). Conversely, depletion of NK cells increased embryo loss in NOD/SCID mice that retained remnants of functional NK cell populations (Lin et al., 2005, 2009c; Parsons et al., 2006).

Regulatory T cells (Treg cells) are known to express functional molecules, including CD25 and fork head box p3/winged helix (Foxp3) and are believed to play a critical role in the induction of allo-immune tolerance (Aluvihare et al., 2004; Saito et al., 2007). The characteristic phenotype of Treg cells is $CD3^+CD4^+CD25^+Foxp3^+$. However, contrasting findings have been recently published. For example, $CD3^-CD49b^+CD25^+Foxp3^+$ cells were found in mice using the magnetic affinity cell sorting (MACS) technique (Lin et al., 2009b; Wang et al., 2009) and $CD4^{low/neg}CD25^+$ Treg cells have been confirmed to have regulatory functions 20 times stronger than that of conventional Treg cells (Vogtenhuber et al., 2008).

1.2. Toll-like receptors (TLRs)

The identification of TLRs as pattern recognition receptors (PRRs) has provided a crucial addition to our understanding of the innate and adaptive immune responses (Medzhitov et al., 1997). Through recognition of pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2000), such as lipopolysaccharide (LPS), flagellin, and double-stranded RNA (dsRNA), TLRs trigger pro-inflammatory and antiviral signaling pathways that ultimately lead to elimination of invading pathogens (Kawai and Akira, 2006).

TLRs are characterized by leucine-rich repeats (LRR) in their extracellular domain that function in ligand binding, and a Toll/IL-1 receptor (TIR) domain in their cytosolic domain that binds to TIR-containing adaptor molecules which regulate downstream signaling (Akira and Takeda, 2004). New members of the TLR family continue to be found, and 13 TLR members have been identified to date in mammals, 10 of which are expressed in humans and 12 in mice (Beutler, 2004; Pandey and Agrawal, 2006). The ligand specificity of many of the mammalian TLRs is still the subject of investigation. For example, TLR11 was recently found to recognize a protozoan profilin-like protein (Yarovinsky et al., 2005).

The cell-surface TLRs, including TLR1, TLR2, TLR4, and TLR6, recognize microbial membrane lipids, whereas TLR3, TLR7, TLR8, and TLR9 recognize pathogen-derived nucleotides in intracellular compartments (Saitoh and Miyake, 2009). TLR7 and TLR9 are sequestered in the endoplasmic reticulum (ER) in a resting state and traffic to endolysosomes upon ligand-induced stimulation. Two molecules in the ER are reported to regulate TLR7/9 trafficking to endolysosomes. PRAT4A (a protein associated with TLR4 A) is associated with TLR9 and is required for ligand-induced trafficking of TLR9 to endolysosomes. UNC93B1 is specifically associated with TLR3, TLR7, TLR9, and TLR13 and regulates ligand-induced trafficking of TLR7 and TLR9 from the ER to endolysosomes (Saitoh and Miyake, 2009).

1.3. Contrasuppression

The original concept of contrasuppression is evident in many immunoregulatory mechanisms. Through inhibition of suppressor activity, contrasuppression may be critical in microbial infection and autoimmunity. The major cellular interactions involved in suppression are the $CD4^+CD25^+$ $Foxp3^+$ Treg cells, programmed death-1 (PD-1):PD-L1/L2 and cytotoxic T lymphocyte antigen-4 (CTLA-4):CD80/86 pathways. These processes are regulated by dendritic cells and a complex array of cytokines of which interleukin (IL)-2, IL-10, IL-6 and transforming growth factor-β (TGFβ) are especially significant. Inhibition of regulatory cells, suppressor pathways and cytokines is consistent with contrasuppression and can be attributed to IL-6, IL-2, IL-10, PD-1, PD-L-1 and TGFβ antibodies, and inhibition of the CTLA-4:CD80/86 and CD40:CD40L pathways. Contrasuppression may regulate innate immunity by TLR expression not only in non-cognate DC, monocytes, NK cells and γδ T cells, but also in adaptive T cells. Furthermore, crosstalk between innate and adaptive immunity may be facilitated by contrasuppressor activity (Lehner, 2008).

2. The confirmed existence of $CD3^-CD49b^+Foxp3^+$ cells

A recent study suggests that IL-10-secreting NK cells purified directly from peripheral blood do not express the Treg cell transcription factor, Foxp3, and express the suppressive cytokine TGFβ in relatively low quantities. These cells appear to be more like IL-10-secreting Tr1 cells than $CD4^+CD25^+Foxp3^+$ Treg cells or TGFβ-secreting Th3 cells (Deniz et al., 2008). Foxp3 is thought to be the specific marker of Treg cells (Zheng and Rudensky, 2007). However, other studies have confirmed the existence of $CD3^-CD49b^+Foxp3^+$ cells in murine spleen, placenta and decidual basalis using MACS (Wang et al., 2009; Lin et al., 2009b) (Fig. 1).

$CD49b^+CD25^+$ cells have been found in the pregnant uterus of NOD/SCID mice, and the dominant NK cell phenotype was determined to be $CD49b^+$, asialo ganglio-N-tetraosylceramide$^+$ $(ASGM1)^+$, $CD25^+$, $CD122^+$, Thy-1 $(CD90)^{hi}$, c-kit $(CD117)^{hi}$, and interleukin-10^+. These NK cells are thought to be critical for the success of pregnancy in these mice, as depletion of NK cells increased the rate of embryo loss (Lin et al., 2009c).

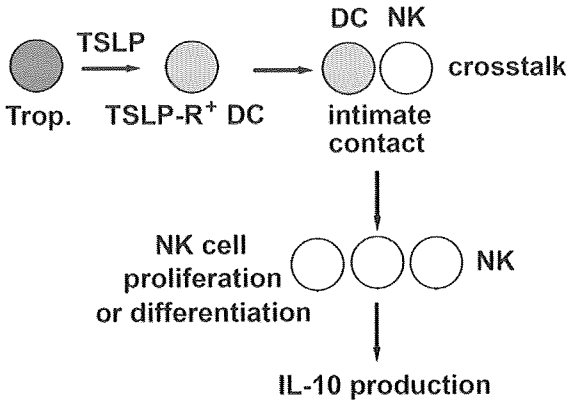

Fig. 1. TSLP–DC–NK signaling. A fraction of trophoblast cells (Trop) express thymic stromal lymphopoietin (TSLP) and activate TSLP receptor positive dendritic cells (TSLP-R$^+$ DCs). The latter cells intimately contact and interact with NK cells and DC–NK crosstalk induces NK cell proliferation or differentiation and increases IL-10 production.

To investigate the possible role that CD25$^+$ NK cells may play in the establishment of pregnancy, CD25$^+$ NK cells from wild-type mice were transferred to subfertile non-obese diabetic (NOD) mice to improve their pregnancy outcome. Molecular production by NK cells was analyzed by flow cytometry before and after the transfer. The proportion of NOD female mice that failed to become pregnant was significantly higher than wild-type control females when co-caged with wild-type males for 16 weeks (53.1% versus 15.1%, $P<0.01$). After transfer of CD25$^+$ NK cells from wild-type mice to the subfertile female NOD mice, an increase in successful pregnancy was observed (77.8% versus 0.0%) in the RMPI-1640 control group and 11.1% in the CD25$^-$CD49b$^+$ NK cell transfer group ($P<0.01$ for both). Improvement of fertility coincided with an increase in the production of Foxp3 by uterine NK cells. Foxp3 expression was confirmed in the CD25$^+$ NK cells before the transfer. These results indicate that subfertility in NOD mice may be partially attributed to inefficient migration of NK cells expressing CD25 and Foxp3 into the uterus during pregnancy, and that these NK cell subgroups may play key roles in the maternal tolerance to a fetus carrying genes from the father (Wang et al., 2009).

In another study, the existence of uterine CD3$^-$CD49b$^+$Foxp3$^+$ cells was confirmed by using MACS-purified CD3$^-$CD49b$^+$ uterine cells and tri-color flow cytometry. Since CD3$^+$ cells were excluded by negative MACS, including any CD3$^+$Foxp3$^+$ regulatory T cells, these CD3$^-$CD49b$^+$Foxp3$^+$ cells are considered a novel NK cell subset (Lin et al., 2009b).

The percentage of CD3$^-$CD49b$^+$Foxp3$^+$ cells in the CD3$^-$CD49b$^+$ population was around 11.5% and 6.4% ($P<0.01$) in allogeneic pregnant BALB/c and NOD mice impregnated by C57BL/6 males, and around 6.4% and 2.8% in syngeneic pregnant BALB/c and NOD mice, respectively. The percentage of CD3$^-$CD49b$^+$ cells in the CD49b$^+$ cell population was 99.8% or higher in all mating combinations. This suggests that most of the CD3$^+$ cells were excluded by negative MACS purification. In addition, the percentage of CD3$^+$Foxp3$^+$ cells in the Foxp3$^+$ cell population was less than 0.2% in all mating combinations. This further confirms that most CD3$^+$Foxp3$^+$ cells are excluded from the Foxp3$^+$ cell population isolated by negative MACS purification (Lin et al., 2009b).

A significantly higher percentage of spontaneous embryo loss was observed in both allogeneic and syngeneic pregnant NOD mice compared to wild-type mice. The percentage of embryo loss in allogeneic pregnant mice was further increased by the administration of anti-ASGM1 to deplete NK cells, but was decreased by adoptive transfer of the CD49b$^+$CD25$^+$ NK cells from wild-type mice. No such trend was observed in syngeneic pregnant NOD mice (Lin et al., 2009b).

3. NK cell migration

The function of T and NK cells and their activation by TLR agonists was further investigated using NOD mice, which are deficient in NK cells and are prone to insulin-dependent diabetes mellitus (IDDM) (Lin et al., 2009d). Using flow cytometry, it was found that fewer peripheral NK cells were detected in NOD mice compared to wild-type mice. This decrease in peripheral NK cells was associated with an increase of bone marrow NK cells, suggesting that a defect in NK cell export from bone marrow to peripheral tissues may be responsible for decreased numbers of peripheral NK cells (Poulton et al., 2001).

The mechanism of NK cell recruitment appears to involve chemokines, such as CXCL8, CCL3 and CX3CL1 (Moretta et al., 2008). Indeed, most classical human NK cells (CD16$^+$CD56^{dim}) express CXCR1 and CX3CR1, while the minor CD16$^-$CD56^{bright} NK subset express CCR7 (Campbell et al., 2001; Cooper et al., 2001; Vitale et al., 2004). On the basis of surface phenotype, it is conceivable that CD16$^+$CD56^{dim} cells may be mainly recruited into pathogen-invaded inflamed tissues, whereas CD16$^-$CD56^{bright} cells may be essentially attracted by secondary lymphoid compartments, such as lymph nodes (Moretta et al., 2008). Indeed, in lymph nodes, CCL21 is highly expressed in high endothelial venules (HEVs), lymphatic vessels, and stromal/interdigitating DCs (predominantly in T-cell areas), whereas CCL19 is highly expressed in mature DCs within the T cell zone of the paracortex (Kawashima et al., 2005).

In a recent study using mouse models, a significantly higher frequency of CXCR4 expression was observed in splenic CD49b$^+$CD25$^+$ NK cells than in splenic CD49b$^+$CD25$^-$ NK cells when purified by MACS. Additionally, this study examined the CXCL12-induced migration of CXCR4$^+$ NK cells using a transwell system. A significantly higher number of CXCR4$^+$ cells was found among the migrated cells compared to the non-migrated cells. This demonstrates the ability of CXCL12 to specifically induce CXCR4$^+$ cell migration, since CXCR4 is the unique receptor of CXCL12 (Lin et al., 2009b). In previous work, CXCL12 expression was confirmed to be expressed by murine trophoblast cells (Lin et al., 2009a). CXCL12-expressing trophoblast cells may preferentially attract CD25$^+$ NK cells to migrate into the pregnant uterus. Therefore, aberrant NK cell homing functions in NOD mice may be partially due to a disorder in the CXCL12–CXCR4 axis (Lin et al., 2009b).

4. NK cell and DC crosstalk

A recent report shows that NK cells require DC for full acquisition of effector function in response to the bacterial-derived TLR9 ligand CpG *in vivo*. DCs were found to play an instrumental role in maintaining normal homeostasis of NK cells. This is achieved through IL-15 production by DCs, which supports the homeostatic proliferation of NK cells (Hochweller et al., 2008). However, some researchers suggest that there is a variable requirement of DCs for recruitment of NK and T cells to different TLR agonists (Uchida et al., 2007). Using mice depleted of more than 90% of $CD11c^+$MHC class II^+ DCs, it was demonstrated that cellular recruitment, including $CD4^+$ T cell and $CD49b^+$ NK cell recruitment to draining lymph nodes following administration of TLR4 and TLR5 agonists to the footpad, is dramatically decreased upon reduction of DC numbers. However, type I IFN production can partially substitute for DCs in response to TLR3 and TLR7 agonists. Interestingly, TLR ligands can activate T cells and NK cells in the draining lymph nodes, even with reduced DC numbers. These findings reveal considerable plasticity in the response to TLR agonists, with TLR4 and TLR5 agonists sharing the requirement of DCs for subsequent lymph node recruitment of NK and T cells (Uchida et al., 2007).

On the other hand, some uNK cells co-localize with immature DC-SIGN$^+$ DCs and this is thought to be critical for the modulation of pregnancy tolerance (Lin et al., 2008; Oh and Croy, 2008).

5. NK cell and TLR signaling

Real-time PCR analysis revealed that TLRs (TLR1-TLR13) are expressed in purified (purity greater than 99%) $CD4^+$ and $CD8^+$ T cells from C57BL/6 and BALB/c mice, where the magnitude of the expression was strain- and cell type-dependent (Salem et al., 2009). In vitro, treatment of these purified T cells with poly(I:C) modulated the expression of TLRs including TLR3 (Salem et al., 2009). Human uNK cells are known to express several TLRs, including TLR2, TLR3 and TLR4 (Eriksson et al., 2006). Other researchers have found that TLR7 is expressed by murine $CD3^-CD49b^+$ NK cells (Sawaki et al., 2007). These facts suggest that TLR ligands may directly interact with TLR-expressing T and NK cells in a DC-independent manner.

Decidual DCs have a unique phenotype that is different from DCs derived from peripheral blood or other extra-uterine tissue, suggesting that these DCs may have unique roles (Degli-Esposti and Smyth, 2005). Earlier work has demonstrated that decidual DCs and uNK cells interact at the feto–maternal interface (Kämmerer et al., 2003). Another study implies that this may be important for thymic stromal lymphopoietin (TSLP)-induced DCs to modulate the differentiation of uNK cells (Lin et al., 2008) (Fig. 1).

We recently investigated the effect of specific TLR3 and TLR7 agonists, poly(I:C) and R837, individually and in combination, on uterine immune cell function and their subsequent effect on allogeneic pregnancy outcome. An additive increase in embryo resorption was observed in wild-type BALB/c mice after induction with both poly(I:C) and R837. Meanwhile, an additive increase of TNFα- and IFNγ-producing cells was detected after the induction of uterine $CD45^+$ cells. Further examination showed that although an additive effect can be detected in both uterine $CD3^+$ cells and $CD49b^+$ cells in BALB/c mice, NOD mouse cells behaved differently. The elevated cytokine staining could be attributed to $CD3^+$ T cells, since no increase was detected in the $CD49b^+$ NK cells of NOD mice. The additive effect of combined agonists was partially inhibited by the Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) inhibitor SP600125 and almost completely abrogated by the extracellular signal-regulated kinase (ERK) MAPK inhibitor PD98059. These results suggest that increased TLR3 and TLR7 signals may be transmitted via Th1-type T cells, rather than NK cells, in NOD mice. Furthermore, the ERK MAPK pathway may be critical in TLR3 and TLR7 signaling (Lin et al., 2009d).

CD69 is an activation marker that triggers NK cytotoxicity (Ntrivalas et al., 2000). The expression of CD69 on peripheral blood NK cells has been related with increased cytotoxicity, and excessive CD69 expression is believed to be harmful to embryo implantation and development (Ntrivalas et al., 2000). In a previous study, CD69 was used as an activation marker for NK cells, and DX5 (CD49b) was used as a pan-NK cell marker. By using these markers, NK cell subsets were analyzed in a poly(I:C)-induced abortion model at the presence or absence of TLR3 blocking. It appeared that upon poly(I:C) stimulation, the resorption rate of embryos was significantly boosted in the absence of anti-TLR3-pretreatment. In contrast, inhibition of TLR3 using neutralizing antibodies completely abrogated the effect of poly(I:C) in the induction of CD69 production in $CD49b^+$ NK cells and the increase of embryo loss. These data imply that poly(I:C) may be involved in the disturbance of embryonic development in mice by interacting with TLR3 and activation of murine NK cells (Lin et al., 2006c). In another report using NK and T cell deficiency NOD/SCID mouse model, TLR3 and poly(I:C) interaction increased the expression of CD80, but failed to change the cytokine profile. In addition, this model appeared to be resistant to poly(I:C)-induced embryo loss. The reason for the relatively low responsiveness of NOD/SCID mice to poly(I:C) stimulation remains unclear. However, it may be due to deficient T cell and NK cell function at the feto–maternal interface (Lin et al., 2006a).

6. LPS-involved TLR signaling

LPS is known to be the specific ligand of TLR4. The finding of increased preterm delivery in IL-10 null mutant mice was first published by Robertson et al. (2006). A recent study reported that low-dose LPS treatment triggered preterm labor and delivery in IL-$10^{-/-}$ but not wild-type mice, in a progesterone-independent manner. Preterm labor and delivery in IL-$10^{-/-}$ mice was associated with an increased number and placental infiltration of cytotoxic uNK cells and placental cell death (Murphy et al., 2009). Using NOD/SCID and wild-type mouse models, our previous study found that LPS interacts with TLR4, triggers the mobilization of $CD45^+CD80^+$ cells, results in elevated production of inflammatory cytokines, and finally results

in preterm delivery. In addition, NK cells may be involved in the signaling cascade, and the lack of functional NK cells in the NOD/SCID may explain why these mice appear to be less sensitive to LPS-induced premature labor (Lin et al., 2006b).

7. Conclusion

Unique cell subsets in pregnant uterus are critical for maternal–fetal tolerance. The migration, differentiation and proliferation of these cells are modulated by chemokines and TLR ligands. A fraction of uterine NK cells express Foxp3 and have a role that is played by both Treg cells and conventional NK cells. TLR ligand stimulation may result in elevated expression of inflammatory cytokines such as IFNγ and TNFα. In normal murine pregnancy, IFNγ plays critical roles that include initiation of endometrial vasculature remodeling, angiogenesis at implantation sites and maintenance of the decidual component of the placenta. Although a certain level of IFNγ is necessary for the success of pregnancy, over-expression of this cytokine is harmful and may result in embryo loss.

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Circulating and Decidual Th17 Cell Levels in Healthy Pregnancy

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Problem
The Th1/Th2 paradigm has recently been reconstituted to include a third population, Th17 cells. It has been reported that Th2 type immunity is predominantly present in normal pregnancy. However, the level of Th17 cells during pregnancy is still unclear. We investigated the level of peripheral Th17 cells in healthy pregnancy subjects.

Method of study
To evaluate the levels of Th17 cells, we investigated the proportion of peripheral blood mononuclear cells that produced IL-17 in the first, second, and third trimester pregnancy subjects using flow cytometry. We further studied the proportion of decidual lymphocytes that produced IL-17 in early pregnant subjects.

Results
Most of the IL-17-producing cells were $CD4^+$ T cells. The number of circulating Th17 cells did not change during pregnancy. In a paired *t*-test of early normal pregnant subjects, the proportion of $IL\text{-}17^+$ decidual lymphocytes was significantly higher than that of peripheral blood lymphocytes.

Conclusion
Th17 levels in peripheral blood lymphocytes do not change during normal pregnancy.

Introduction

Wegmann et al. proposed that Th2 cytokines may play an important role in the maintenance of murine pregnancy by inhibiting Th1 responses that induce pregnancy failure.[1] His hypothesis has since been adopted in the maintenance of normal human pregnancy,[2,3] pregnancy failure such as recurrent spontaneous abortion,[4,5] and pre-eclampsia.[6,7] However, recent data on cytokine expression at the materno–fetal interface indicated some problems with this hypothesis.[8] The Th1/Th2 paradigm has recently been reconstituted to include a third population of T helper cells that produce IL-17, which are designated as Th17 cells.[9–11] Th17 cells have specific roles in host defense against extracellular bacteria and fungi[12] and play an important role in the induction of autoimmune diseases.[9–11] Recent data revealed that transforming growth factor (TGF)-β is able to induce the differentiation of regulatory T (Treg) cells and that the combination of the pro-inflammatory cytokine IL-6 and TGF-β is able to induce the differentiation of Th17 cells from naïve T cells *in vitro* in mice,[13] although the processes behind the induction and regulation of Th17 cells in humans are different from those in mice.[11] Recent data show that Treg cells play a very important role in the maintenance of pregnancy,[14,15] and decreased numbers of Treg cells have been reported in abortion and pre-eclampsia.[16–18] An imbalance between Th17