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.

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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.,
2. Materials and methods

2.1. Reagents

TLR7 agonist imiquimod (1-(2-methylpropyl)-1H-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 mm³) with ocular scissors. The pieces then were collected in Hank’s buffered salt solution (NaCl, 8 g; KCl, 0.4 g; CaCl₂, 0.14 g; MgSO₄, 0.2 g; Na₂HPO₄, 0.06 g; NaHCO₃, 0.35 g; and glucose, 1 g in 11 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, as described above. 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 ⁵¹Cr 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. ⁵¹Cr-labeled (PerkinElmer, Boston, MA) YAC-1 target cells (5 × 10³ 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 ⁵¹Cr-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 10⁶ 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

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 10⁶ 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 pooled placentas from each mouse were carefully cut into small pieces (<1 mm³) with ocular scissors. The pieces then were collected in Hank’s buffered salt solution (NaCl, 8 g; KCl, 0.4 g; CaCl₂, 0.14 g; MgSO₄, 0.2 g; Na₂HPO₄, 0.06 g; NaHCO₃, 0.35 g; and glucose, 1 g in 11 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, as described above. 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.

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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 χ² 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).

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 \textit{in vivo} poly (I:C) and R837 stimulation on the increase of intracellular TNF-\(\alpha\) and IFN-\(\gamma\) production in placental leukocytes. The percentage positive for the indicated cytokines in the CD45\(^+\) cell population was compared. (A) TNF-\(\alpha\); (B) IFN-\(\gamma\); (C) IL-4; (D) TGF-\(\beta\); 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.

\(^b\)\(P < 0.01\) 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.

*P < 0.01 between different treatments within the same group 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 ⁵¹Cr 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 ± 1.0%, 16.4 ± 1.1% and 25.1 ± 1.4% 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 ± 0.8% (P < 0.01), 11.5 ± 0.9% (P < 0.05) and 19.4 ± 1.5% (P < 0.05), respectively. Addition of PD98059 decreased the percentages further to 7.6 ± 0.5%, 8.3 ± 0.5% and 10.8 ± 1.0%, 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 ± 1.4%, 18.0 ± 1.3%, and 36.2 ± 1.9% 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 16.1 ± 0.9% (P < 0.01), 15.8 ± 1.2% (P < 0.05) and 25.8 ± 1.6% (P < 0.05), respectively. Addition of PD98059 decreased the percentages further to 10.5 ± 0.5%, 11.2 ± 0.6% and 13.7 ± 1.1%, 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).

Fig. 4. CD49b⁺ NK cell cytotoxicity detected by ⁵¹Cr 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 placenta 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.

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

aP<0.01 between different treatments within the same group of mice.
stimulation group, respectively. Addition of SP600125 decreased these percentages to 19.5 ± 1.1% (P > 0.05), 17.5 ± 1.2% (P > 0.05) and 29.3 ± 1.2% (P < 0.05), respectively. Addition of PD98059 decreased these percentages to 6.5 ± 0.9%, 7.0 ± 0.8%, and 16.5 ± 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 uterus. 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 therefore, that T cells are responsible for promoting 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.

Some recent reports 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.

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