Anakinra and etanercept prevent embryo loss in pregnant nonobese diabetic mice

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Abstract

Bacteria and viruses activate the host innate immune response via Toll-like receptor (TLR)-involved signaling and potentially cause pregnancy failure. TLR7 and TLR9 respond to single-stranded RNA (a viral intermediate) and hypomethylated CpG DNA motifs (specific molecular constituents of bacteria) respectively. In this study, we treated murine RAW264.7 cells with R837, CpG1826, or a combination of the two. RT-PCR was performed to detect cytokines, *Tlr7*, and *Tlr9*. WT and nonobese diabetic murine embryo resorption models were established by i.p. injections of TLR7 and TLR9 ligands. Neutralizing antibodies and the IL1 β and TNF α inhibitors were used. The specific inhibitors anakinra and etanercept effectively prevented TLR7 and TLR9 ligand-induced embryo loss. Notably, this effect was not observed in decidual NK cell-depleted mice. Our findings suggest that anakinra and etanercept may have potential for preventing TLR7 or TLR9 ligand-induced abortion in the presence of decidual NK cells.

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Introduction

Considerable progress has been made in overcoming obstetric problems. However, preventing and reducing pregnancy failure remain as a major challenge. Approximately 10–15% of pregnant women undergo miscarriages that occur up to 24 weeks of gestation (Oliver & Overton 2014). Many studies have attempted to study the etiopathogenesis of pregnancy failure, and microbial infection has emerged as an important cause of pregnancy failure in many of these studies. A large body of evidence indicates that inflammatory cytokines and chemokines are involved in bacterial and viral infections (Lin *et al.* 2009a, Ilievski & Hirsch 2010, Li *et al.* 2012). Further data highlighting the significant relationship between Toll-like receptors (TLRs) and adverse pregnancy outcomes have drawn considerable attention (Filipovich *et al.* 2009, Lin *et al.* 2009b,c, 2014, Sun *et al.* 2013).

The innate immune system defends against invading microorganisms through pattern-recognition receptors such as TLRs (Takeda & Akira 2004). TLRs can identify an extensive range of pathogen ligands including bacterial, viral, fungal, and protozoan components (Takeda & Akira 2004, Kawai & Akira 2005). TLRs are expressed by a wide range of immunocytes as well as nonimmune cells, and their expression is influenced by pathogens, cytokines,

and outside stresses (Akira *et al.* 2006). Thus far, 13 TLR family members, distributed in different cellular compartments, have been discovered in mammals. TLR1, TLR2, TLR4, TLR5, and TLR6 are found mainly on the cell surface, while TLR3, TLR7, TLR8, and TLR9 are almost exclusively expressed within the cells (Kawai & Akira 2005, Akira *et al.* 2006, Ilievski & Hirsch 2010).

Each TLR has a specific ligand. During viral infection, TLR7 recognizes single-stranded RNA, which is the intermediate generated during viral replication (Heil *et al.* 2004). TLR9 mediates cellular responses to bacteria via their CpG motifs (Krieg 2002, Pedersen *et al.* 2005). Once TLR is stimulated by a ligand, downstream signaling events activate certain transcription factors to produce a series of inflammatory cytokines as the host defense response (Takeda & Akira 2004).

Nonobese diabetic (NOD) mice have low fertility and are susceptible to TLR-induced embryo loss (Gautier *et al.* 2005, Lin *et al.* 2009f, Wang *et al.* 2009). Some studies have demonstrated that NOD mice exhibit insufficient numbers of NK cells migrating to the pregnant uterus (Wang *et al.* 2009, Sun *et al.* 2013, Lin *et al.* 2014). As decidual NK cells are considered to be a key cell subset beneficial for pregnancy success (Lin *et al.* 2006a, 2014, Wang *et al.* 2009, Sun *et al.* 2013), we used an NK cell-deficient NOD

model and a WT control (Wang *et al.* 2009, Sun *et al.* 2013). In this study, we aimed to investigate the effects of combined stimulation of TLR7 and TLR9, in order to mimic a mixed infection with viruses and bacteria, both *in vitro* and in a murine pregnancy model.

Materials and methods

Mice and ethics

Female Balb/c, NK cell-deficient female NOD and male C57BL/6 mice (8–12 weeks of age; body weight, 18–22 g) were obtained from the Model Animal Center of Nanjing University (Nanjing, China). All the mice were housed in a pathogen-free facility. Balb/c×C57BL/6 and NOD×C57BL/6 mating combinations were established naturally, and the pregnant mice were treated to establish the induced embryo resorption models as described in the following sections. Samples were randomized as described previously (Lin *et al.* 2009d). The immunodeficiency of NOD mice was confirmed using the methods described previously (Lin *et al.* 2005, Rocha-Campos *et al.* 2006). All animal procedures were carried out in accordance with national animal care guidelines, and the study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Wuhan University. All related data were approved for publication by the Institutional Review Board and Ethics Committee of Wuhan University. Each experimental group contained at least four mice. The day of vaginal plug appearance was designated as gestational day 0.5 (E0.5; Lin *et al.* 2006a, Sun *et al.* 2013).

Cell culture

The mouse macrophage cell line RAW264.7 (American Type Culture Collection, Manassas, VA, USA; TIB-71) was cultured in DMEM (high glucose; 11965-092; Gibco) supplemented with 10% fetal bovine serum (FBS), 1% streptomycin, and 1% penicillin in tissue culture flasks at 37 °C in 5% CO₂ and 95% air. The cells were passaged every 2–3 days to ensure logarithmic phase growth. Before each experiment, the cells (4×10⁵ cells/well) were plated in six-well plates in triplicate, incubated for 2 h, and then treated with PBS, R837 (5 µg/ml), or CpG1826 (10 µg/ml) or both R837 and CpG1826 (both from Invivogen, San Diego, CA, USA). For sequential incubations, the medium was removed and the cells were washed three times with PBS before incubation with a second reagent. All tissue culture experiments were performed in triplicate and repeated three times (Lin *et al.* 2006a, Ilievski & Hirsch 2010, Sun *et al.* 2013).

We used Trypan Blue dye exclusion to assess the viability of the cultured RAW 264.7 cells. The viability before plating for trial was 96%. The viability at 5 h after plating was 94% for the control (medium), 92% for R837, 93% for CpG, and 92% for R837 plus CpG. The post-plating cell viability did not significantly differ between the groups (Ilievski & Hirsch 2010).

RT-PCR analysis

We evaluated the activation of TLR signaling pathways using RT-PCR system by measuring the relative quantity of transcripts

such as interleukin 1 beta (*Il1b*), nitric oxide synthase 2 (*Nos2*), chemokine CCL5 (*Ccl5*), tumor necrosis factor alpha (*Tnf*), *Tlr7*, and *Tlr9* (Ilievski & Hirsch 2010). At the end of the tissue culture experiment, the medium was aspirated, and the cells were washed once with PBS and lysed with TRIzol reagent (Invitrogen) to extract total RNA according to the manufacturer's protocol. All PCR primers and probes were purchased from Applied Biosystems (*Il1b*, Mm00434228; *Nos2*, Mm00440485; *Ccl5* (Rantes), Mm01302428; *Tnf*, Mm00443258; *Tlr7*, Mm00446590; *Tlr9*, Mm00446193; and mouse glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*; 203, 4452339E). TaqMan PCR reagent kits were used in accordance with the manufacturer's manual. Reactions occurred in a 10-µl mixture containing 0.5 µl of cDNA. Duplex RT-PCR was performed with one primer pair amplifying the gene of interest and the other amplifying an endogenous reference gene (*Gapdh*) in the same tube. The thermocycler parameters were 50 °C for 2 min, 95 °C for 10 min, and 30–45 cycles at 95 °C for 15 s and 60 °C for 1 min. Semiquantitative analysis of gene expression was performed using the comparative CT method, normalizing the expression of the gene of interest to that of *Gapdh*. PCR assays were performed in duplicate for each of the triplicate tissue culture samples (Ilievski & Hirsch 2010).

In vivo treatment of pregnant mice

R837 is a specific agonist for TLR7 (Heil *et al.* 2004, Lin *et al.* 2009b) and CpG is a specific agonist for TLR9 (Krieg 2002, Pedersen *et al.* 2005). The WT and NOD female mice impregnated by C57BL/6 males received i.p. injections of 12.5 µg R837 or 25 µg of CpG oligodeoxynucleotide (CpG1826; Invivogen) on E6.5 (Thaxton *et al.* 2009, Sun *et al.* 2013).

For the neutralizing antibody experiment, mice were intra-peritoneally administered 40 µg/dam monoclonal anti-IL1β Ab (B122; BioLegend, San Diego, CA, USA) and 250 µg/dam anti-TNFα Ab (Gr81-2626; BD Pharmingen, San Diego, CA, USA) on E5.5 and R837 and CpG injections on E6.5 (Thaxton *et al.* 2009, Sun *et al.* 2013).

Anakinra is a specific inhibitor for IL1β and etanercept is a specific inhibitor for TNFα (McCall *et al.* 2012). In the anakinra and etanercept experiments, the pregnant mice received i.p. injections of 100 µg/dam control IgG, 300 µg/dam anakinra, 100 µg/dam etanercept, or a combination of anakinra and etanercept at the mentioned doses (McCall *et al.* 2012, Johnston *et al.* 2014).

For the NK cell function experiment, NK cell depletion was performed on E4.5, E6.5, and E8.5 using 50 or 100 µl of anti-asialo GM1 (ASGM1), or an equal volume of nonimmune rabbit serum (Wako, Osaka, Japan) (Lin *et al.* 2006b, Thaxton *et al.* 2009, Sun *et al.* 2013).

Pregnant mice were killed on E10.5 and the embryo resorption rate (%) was calculated as follows: (number of resorbed embryos)/(total number of embryos)×100 (Lin *et al.* 2006a).

Cell preparation

Uterine mononuclear and granular cells were isolated by mincing and mechanical dispersion of whole E10.5 uteroplacental tissue in RPMI-1640 supplemented with 10% FBS,

penicillin/streptomycin, and L-glutamine. Single-cell suspensions from uterine horns were sifted through a 100- μ m cell strainer followed by density gradient separation using Fico/Lite-LM (Atlanta Biologicals, Flowery Branch, GA, USA). These experiments were performed on the three layers obtained from Ficoll gradient separation to determine the layer in which the granulocytes accumulated. Granulocytes were found to get collected directly below the monocyte layer, and both layers were harvested together for further experiments (Thaxton *et al.* 2009, Sun *et al.* 2013).

ELISA

As no suitable NOS ELISA kit was available, serum IL1 β , CCL5, and TNF α cytokine levels were measured on E10.5. Briefly, peripheral blood was harvested from vena orbitalis (Lin *et al.* 2005), allowed to clot for 30 min at room temperature, spun at 8000 r.p.m. for 20 min at 4 °C, and supernatants were collected and frozen for further analyses. IL1 β , CCL5, and TNF α were assayed using Quantikine ELISA kits (catalog number: SMLB00C, MMR00, and SMTA00B respectively; R&D Systems, Minneapolis, MN, USA) and experiments were performed according to the manufacturer's instructions. Separate serum samples were collected from each experimental treatment group ($n=6$ /group; Thaxton *et al.* 2009).

Flow cytometry

Antibodies specific for CD45 (30-F11) were purchased from BD Biosciences, Franklin Lakes, NJ, USA. FITC-conjugated *Dolichos biflorus* agglutinin (DBA)-lectin was purchased from Sigma-Aldrich. Isotype controls were established by staining of isotype control Abs to exclude false-positive cells (Lin *et al.* 2006a, 2009a).

Statistical analysis

Based on a normality test, the Student's *t*-test was used to evaluate differences in the gene expression between groups. For comparison of multiple variables, ANOVA was used. Differences were considered to be statistically significant when $P<0.05$ (Ilievski & Hirsch 2010, Li *et al.* 2012).

Results

Effect of R837 and CpG1826 on *Il1b* and *Tnf* production

The mouse macrophage cell line RAW 264.7 was used for our *in vitro* experiment. Both R837 and CpG1826 could synergistically enhance *Tlr7* and *Tlr9* activation and boost *Il1b* and *Tnf* production in RAW 264.7 cells (Fig. 1). Challenge with either R837 or CpG1826 alone increased the *Il1b* (ninefold for R837 and eightfold for CpG) and *Tnf* production (eightfold for R837 and tenfold for CpG) (Fig. 1). After challenge with R837 and CpG1826 together, the *Il1b* and *Tnf* production was synergistically increased by 25- and 33-fold, respectively, compared with the controls (Fig. 1A and D).

Figure 1 Synergistic effect of R837 and CpG on *Il1b* and *Tnf* production. RAW264.7 cells were stimulated with either PBS or R837 or CpG or a combination of R837 and CpG for 5 h. RT-PCR was performed for *Il1b* (A), *Nos2* (B), *Ccl5* (C), *Tnf* (D), *Tlr7* (E), and *Tlr9* (F), and the results were normalized to the housekeeping gene *Gapdh*. NS, not significant. * $P<0.01$.

No change was observed in TLR7- and TLR9-modulated *Nos2* and *Ccl5* production (Fig. 1B and C). The *Tlr7* expression level significantly increased upon R837 stimulation, and the *Tlr9* expression was significantly increased upon CpG1826 stimulation. However, the *Tlr7* and *Tlr9* levels showed no significant changes following combined treatment with R837 and CpG1826 (Fig. 1E and F). This result implies that the synergistic effect of R837 and CpG on *Il1b* and *Tnf* mRNA production depends on downstream events in the TLR7 and TLR9 signaling pathways.

TLR7 or TLR9 engagement can prime cells for synergistic activation via the alternate ligand

We examined whether the synergistic interaction between TLR7 and TLR9 requires presence of both the ligands. The RAW264.7 cells underwent sequential stimulation by incubation with R837 or CpG1826 for 5 h, followed by washing and incubation for an additional 5 h with the alternate ligand. The results were compared with those obtained by exposing the cells to either one or both ligands for 5 and 10 h. Sequential stimulation with either R837 followed by CpG1826 or *vice versa* induced the synergistic expression of both *Il1b* and *Tnf* mRNAs, similar to that observed after simultaneous stimulation for *Tnf* mRNA, but displays a lower level than simultaneous stimulation for *Il1b* (Fig. 2A and D). Meanwhile, no such effect was observed in the modulation of *Nos2* or *Ccl5*, which is the same as the result obtained with simultaneous stimulation (Fig. 2).

Figure 2 Effects of continuous and sequential R837 and CpG stimulations on *Tlr7* and *Tlr9* activation. RAW264.7 cells were treated in cell culture systems with either R837 or CpG or both R837 and CpG either simultaneously or sequentially for 5–10 h. For sequential incubations, the cultures were incubated with the first reagent for 5 h, washed with PBS, and incubated for an additional 5 h with the second reagent. RT-PCR was performed using *Gapdh* as the control gene. NS, not significant. * $P < 0.01$.

Effects of IL1 β and TNF α inhibitors on murine embryo resorption

We used neutralizing Abs for IL1 β and TNF α to examine their effects on R837- and CpG1826-induced embryo resorption in WT and NOD mice. We found that single antibodies against IL1 β and TNF α could not decrease embryo resorption to a statistically supported level. Administration of combined antibodies for IL1 β and TNF α significantly decreased the embryo resorption rate on the indicated doses (45.0% vs 24.5% in WT and 70.5% vs 35.6% in NOD; $P < 0.05$ for both; Fig. 3A and B).

Anakinra and etanercept were used to inhibit embryo resorption induced by R837 and CpG1826 (McCall *et al.* 2012). Using a single inhibitor significantly decreased the level of the TLR7 and TLR9 ligand-induced embryo resorption. Challenge with both inhibitors in combination (anakinra plus etanercept) further decreased the embryo resorption rate (44.3% vs 6.6% in WT and 75.0% vs 17.5% in NOD; $P < 0.01$ for both; Fig. 3C and D). In other words, almost completely abrogated R837 and CpG1826 induced embryo resorption increase (6.6% vs 5.2% in WT and 17.5% vs 18.2% in NOD; Fig. 3C and D).

Figure 3 Effects of IL1 β and TNF α inhibitors on murine embryo resorption. (A and B) Neutralizing antibodies against IL1 β and TNF α . (C and D) Anakinra (A) and etanercept (E) were used as the specific inhibitors for the IL1 β and TNF α receptors respectively. These antibodies or inhibitors were used to prevent the R837 plus CpG-induced increase in murine embryo resorption. Single anti-IL1 β or anti-TNF α antibodies did not change the embryo resorption rate. However, combined treatment of anti-IL1 β and anti-TNF α antibodies significantly decreased the embryo resorption rate in both WT and NOD mice. In comparison, anakinra or etanercept when used individually at the indicated doses significantly decreased the R837- and CpG-induced increases in embryo resorption, while the combined inhibition using both inhibitors (anakinra plus etanercept or A + E) completely abrogated the R837- and CpG-induced increase in embryo resorption. NS, not significant. * $P < 0.05$ and ** $P < 0.01$.

Cytokines IL1 β and TNF α but not CCL5 were increased by combined R837 and CpG stimulation

As examined by ELISA, serum IL1 β (262.0 ± 32.2 pg/ml vs 37.8 ± 12.3 pg/ml in WT and 272.7 ± 33.7 pg/ml vs 24.3 ± 7.9 pg/ml in NOD; $P < 0.01$ for both) and TNF α (697.3 ± 105.9 pg/ml vs 179.5 ± 48.5 pg/ml in WT and 592.3 ± 141.4 pg/ml vs 186.2 ± 61.2 pg/ml in NOD; $P < 0.01$ for both) cytokine levels were significantly increased upon combined R837 and CpG stimulation in both WT and NOD mice (Fig. 4A and C). In contrast, no such effect was found in chemokine CCL5 level (45.3 ± 14.3 pg/ml vs 53.0 ± 8.7 pg/ml in WT and 42.5 ± 13.8 pg/ml vs 42.0 ± 13.4 pg/ml in NOD; not statistically significant for both) (Fig. 4B). In addition, no significant difference was found in serum IL1 β and TNF α cytokine levels between WT and NOD mice (Fig. 4).

Anakinra and etanercept prevent R837- and CpG-induced embryo loss in an NK cell-dependent manner

ASGM1 is a specific inhibitor of murine NK cells, which is commonly used to deplete decidual NK cells (Lin *et al.* 2006b, 2009d). In this study, combined treatment with anakinra and etanercept resulted in a potent decrease in the R837- and CpG-induced embryo resorption rate from 42.5 to 9.3% ($P < 0.01$) in control mice, but this effect was diminished after ASGM1 treatment (from 50.0 to 37.5%, $P > 0.05$). Similar results were obtained in NOD mice: combined treatment decreased the R837- and CpG-induced resorption rate from 70.0 to 20.9% ($P < 0.01$); this effect was weakened in the ASGM1 group (100.0–60.0%, $P > 0.05$; Fig. 5). Our study indicated that combined treatment of anakinra and etanercept could prevent the R837- and CpG-induced embryo loss in an NK cell-dependent manner.

Discussion

Bacteria and viruses activate the innate immune responses of the host via TLR-mediated signaling. Infection by single or multiple pathogens may result in pregnancy failure. However, the mechanisms involved in this process remain largely unclear. TLR7 and TLR9 specifically respond to single-stranded RNA and CpG, which represent viral and bacterial infections respectively. Our results indicate their synergistic effect in mediating proinflammatory response, as combined stimulation with both the ligands resulted in a higher level of toxic cytokines than when a single ligand was used. Previous research exploring bacterial and viral coinfection has shown synergistic interaction between TLR2 and TLR3 (Ilievski & Hirsch 2010). Furthermore, the additive effect of TLR3 and TLR7 cross-talk on immune cell function and their subsequent effects on pregnancy were explained (Lin *et al.* 2009b). In another study, TLR2 responded to many other TLR ligands, and its expression was upregulated significantly in response to different

Figure 4 Cytokines IL1 β and TNF α but not CCL5 were increased upon combined R837 and CpG stimulation. Pregnant mice were i.p. injected with R837 and CpG1826 combination on E6.5 and killed on E10.5 to detect the serum cytokine level by ELISA. (A) IL1 β . (B) CCL5. (C) TNF α . IL1 β and TNF α cytokine levels were significantly increased upon combined R837 and CpG stimulation, while no significant difference was found in the chemokine CCL5 level. NS, not significant. * $P < 0.01$. $n = 6$ per group.

microbial products (Nilsen *et al.* 2004). Therefore, there should be a greater focus on the prevention of multiple infections during pregnancy in clinical practice. In addition, the effect of occult infections may be underestimated for the superimposed effect between different kinds of pathogen.

NOD mice are much more sensitive to TLR7 and TLR9 ligands than WT mice (Sun *et al.* 2013, Lin *et al.* 2014). Inhibition of remnant NK cells in NOD mice will further increase the percentage of embryo loss (Lin *et al.* 2009d). In a previous study, NK cells in the pregnant uterus, featured as CD16⁺CD56^{bright}, were found to have little cytolytic activity while producing high levels of cytokines. In contrast, CD16⁺CD56^{dim} NK cells, which were predominately distributed in the peripheral blood, produced lower levels of cytokines but exhibited more cytotoxicity (Lin *et al.* 2009e). These findings indicate that the NK subsets in the pregnant uterus may play a supportive role in maintaining a successful pregnancy. In another study, transferring the CD25⁺CD49b⁺ subset of NK cells to NOD mice was found to improve pregnancy outcomes (Wang *et al.* 2009).

In this study, we found that costimulation of mouse RAW 264.7 cells with both R837 and CpG resulted in synergistic expression of mRNA for *Il1b* and *Tnf* compared with either R837 or CpG alone. Then, sequential stimulation was performed by incubating RAW 264.7 cells with either R837 or CpG for 5 h, followed by washing and incubation for an additional 5 h with the alternate ligand. These conditions were compared with both 5 and 10 h of simultaneous single- and double-ligand exposure. It showed that sequential stimulation with either R837 followed by CpG or *vice versa* induced synergistic expression of the same mRNAs for *Il1b* and *Tnf* as observed in simultaneous stimulation settings. However, the level of increase was similar to simultaneous stimulation only for *Tnf* mRNA, while the level of increase was significantly lower than simultaneous stimulation for *Il1b* mRNA. The mechanism for this is unclear. It implies a lower efficacy in *Il1b* mRNA induction in sequential stimulation conditions.

Anakinra and etanercept are specific receptor antagonists of IL1 β and TNF α respectively (McCall *et al.* 2012).

Although they have been used clinically to treat inflammatory diseases like rheumatoid arthritis, little is known about their role in pregnancy (McCall *et al.* 2012). Neutralizing Abs slightly decreased the embryo resorption rate induced by R837 and CpG. However, anakinra and etanercept made the R837- and CpG-induced increase in embryo resorption to decline almost completely. These findings suggested that anakinra and etanercept may be useful to prevent pregnancy failure in cases of combined bacterial and viral infection (Chen *et al.* 1998, McCall *et al.* 2012, Guo *et al.* 2013).

When decidual NK cells were inhibited by the specific NK cell inhibitor ASGM1, anakinra and etanercept could not prevent embryo resorption. This indicates that the mechanism by which anakinra and etanercept prevent embryo loss depends on the presence of decidual NK cells (Wang *et al.* 2009).

However, we have not illustrated the mechanism of this interesting NK-dependent function in our study. Hanna *et al.* (2006) suggest that decidual NK cells, but not peripheral blood-derived NK subsets, regulate trophoblast invasion both *in vitro* and *in vivo* by production of the IL8 and interferon-inducible protein 10 chemokines, and that decidual NK cells are potent secretors of an array of angiogenic factors and induce vascular growth in the decidua. The finding implies that inhibition of decidual NK cells by ASGM1 itself may result in pregnancy failure by other mechanisms, such as impaired trophoblast invasion, interfered production of angiogenic factors, and aberrant vascular growth, instead of modulating IL1 β - and TNF α -involved inflammation.

Both IL1 β and TNF α are important devastating inflammatory factors. IL1 β regulates the expression of several genes such as *Il6* and *Tnf* through its receptor. TNF α , on the other hand, binds to cellular death domain and kills cells (McCall *et al.* 2012). IL1 β and TNF α may work together to damage decidual NK cells. In this study, the serum levels of IL1 β and TNF α cytokines were found to be markedly upregulated by combined R837 and CpG stimulation in pregnant mice on E10.5, in line with the RT-PCR results. Anakinra and etanercept may primarily inhibit the binding of these factors to decidual NK cells. Without these cells, anakinra and etanercept will not be able to function as described, leading to a high rate of pregnancy failure, as shown in our study.

Acute infection within the gestational compartment may be life threatening for pregnant women. Therefore, infection-induced abortion can be considered a strategy by which the host evacuates an infected body cavity, thus ensuring her own survival with retention of future reproductive potential (Thaxton *et al.* 2009, Ilievski & Hirsch 2010).

The presence of viral pathogens within the gestational compartment and their effects are possibly underestimated in clinical practice because of limitations in viral culture and molecular methodology. Therefore, viral infection may potentially play a hitherto

Figure 5 Anakinra and etanercept prevent R837- and CpG-induced embryo loss in an NK cell-dependent manner. (A and B) Double-challenged samples that were challenged with both R837 and CpG1826. ASGM1, anti-asialo GM1 antiserum. A, anakinra; E, etanercept; A + E, anakinra plus etanercept. When decidual NK cells were depleted by ASGM1 injection, administration with A, E, or A + E failed to inhibit the TLR7 and TLR9 ligand-induced increase in embryo resorption in both the WT and NOD mice. The effect of ASGM1 on decidual NK cell inhibition was confirmed in WT mice (C, D, E and F) and NOD mice (G, H, I and J) using flow cytometry. A small number of remnant NK cells were detected in some cases (H). DBA-lectin was used as a specific marker for murine decidual NK cells. M, Maximum embryo resorption rate (100%) was observed under the indicated condition. NS, not significant. * $P < 0.01$.

underappreciated role in pregnancy failure in culture-negative cases. Under the presence of subclinical viral infection, an acute and robust immune response may be triggered once there is an additional bacterial infection (Lin *et al.* 2009b, Ilievski & Hirsch 2010).

In some cases, endogenously produced TLR ligands may influence the pregnancy outcome. At least two such candidate endogenous TLR ligands are potentially relevant to pregnancy outcomes, namely, low-molecular-weight hyaluronan (a component of cervical extracellular matrix degraded during cervical ripening) and surfactant protein A (a protein produced by the maturing fetal lung that acts as a hormone signaling the onset of parturition in mice) (Condon *et al.* 2004, Jiang *et al.* 2005, Ilievski & Hirsch 2010).

In conclusion, our findings reinforce the growing body of evidence demonstrating the key influence of TLRs on pregnancy outcomes. As *Il1b* and *Tnf* mRNAs were synergistically increased following stimulation by R837

and CpG, the specific inhibitors anakinra and etanercept are potential candidate agents in the prevention of viral and bacterial causes of pregnancy failure.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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