Adoptive transfer of pregnancy-induced CD4+CD25+ regulatory T cells reverses the increase in abortion rate caused by interleukin 17 in the CBA/J × BALB/c mouse model

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STUDY QUESTION: Could adoptive transfer of pregnancy-induced CD4+CD25+ regulatory T cells (Tregs) reverse the increase in abortion rate caused by interleukin 17 (IL-17) in the CBA/J × BALB/c mouse model?

SUMMARY ANSWER: The effects of exogenous IL-17 on increased abortion rate, as well as decreased transforming growth factor (TGF)-β and IL-10 expression, are reversed by a pre-mating transfusion of Tregs in a mouse model of pregnancy.

WHAT IS KNOWN ALREADY: IL-17 is a pro-inflammatory cytokine mainly expressed by T helper 17 cells, and plays a pivotal role in the pathogenesis of endometriosis, miscarriage, preterm labor and pre-eclampsia. The activity of Th17 cells is attenuated by the anti-inflammatory action of Tregs.

STUDY DESIGN, SIZE, DURATION: Fifty microliters of phosphate-buffered saline (PBS) (Group 1,) or recombinant IL-17 (rIL) (10 μg/mouse) supernatant (Group 2) was administered in the vaginal vaults of anesthetized pregnant CBA/J mice on Day 1 of pregnancy. Tregs (2 × 10^5 cells) purified from pregnant CBA/J × BALB/c mice were given i.v. via the tail vein 2 days before mating (Group 3) or on Day 7 of pregnancy (Group 4).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Mice (n = 40) were randomly assigned to one of four experimental groups. The numbers of surviving and reabsorbed fetuses in each group were counted on Day 14 of pregnancy, and the expression of interferon (IFN)-γ, IL-4, TGF-β and IL-10 in the decidual tissue was assessed by real-time RT–PCR and western blotting.

MAIN RESULTS AND THE ROLE OF CHANCE: Normal pregnant CBA/J mice mated with BALB/c males which received transvaginal rIL-17 presented with a significantly increased abortion rate compared with the group which received PBS (27.7 versus 9.9%, respectively; P < 0.05). The transfusion of pregnancy-induced Tregs from 14-day normal pregnant mice 2 days before mating reduced the abortion rate caused by IL-17 (12.5 versus 27.7%, respectively; P < 0.05), while transfusion of Tregs on Day 7 of pregnancy had no effect. Transfusion of Tregs did not affect IFN-γ or IL-4 expression in the decidual tissue at either the mRNA or protein level. Administration of rIL-17 resulted in a decrease in production of TGF-β and IL-10 at both mRNA and protein levels (P < 0.05). Transfusion of Tregs before mating increased TGF-β and IL-10 mRNA and protein levels (P < 0.05), while Tregs transfusion at Day 7 of pregnancy had no effect on TGF-β or IL-10 expression.

LIMITATIONS, REASONS FOR CAUTION: These data derive from only a small number of mice. It is unclear whether the same effects would be seen in humans.

WIDER IMPLICATIONS OF THE FINDINGS: Abnormally elevated expression of IL-17 in the feto-maternal interface may result in miscarriage. Transfer of antigen-specific Tregs before mating takes place may have potential applications in the prevention of recurrent spontaneous abortion.

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Introduction

The human conceptus is a semi-allograft and hence antigenically foreign to the mother. The process of implantation may therefore include mechanisms to prevent allograft rejection, but once immunological tolerance becomes imbalanced, pathological pregnancy, including spontaneous abortion, may occur (Medawar, 1953; Trowsdale and Betz, 2006). Local mechanisms may play an important role in the evasion of immune attack (Sunder and Lenton, 2000). CD4 + T cells, which include T helper 1 (Th1), Th2, regulatory T cells (Tregs) and Th17 cells, play an important role in the fetomaternal immune response.

Regulation of the Th1/Th2 cytokine balance has been seen as an important mechanism determining the survival of the fetus in the maternal uterus in humans and murine (Linet et al., 1993; Raghupathy, 1997). The production of Th2-type cytokines, such as interleukin (IL)-4, locally at the fetomaternal interface favors the maintenance of mammalian pregnancy (Piccinni et al., 1998), while Th1-type cytokines, such as interferon (IFN)-γ, mediate fetal rejection (Zenclussen et al., 2001). However, IFN-γ is essential for implantation both in humans and mice (Jokhi et al., 1994; Ashkar et al., 2000), and genetically IL-4-deficient mice do not show disturbed pregnancy (Svensson et al., 2001), thereby challenging the Th1/Th2 concept of fetomaternal immunological tolerance. Tregs participate in the suppression of maternal alloresponses targeted against the fetus, maintaining the pregnancy by actively suppressing self-reactive lymphocytes via mechanisms mediated by a cell–cell contact and production of the soluble factors transforming growth factor (TGF)-β and IL-10. Defects in the numbers or functions of Tregs have been documented in miscarriage in humans and murine (Aluvihare et al., 2004; Sasaki et al., 2004). Th17 cells secrete their signature cytokine, IL-17, share a complex relationship with Tregs and thereby affect the survival of transplants maintained by Tregs (Harrington et al., 2005). Abnormal expression of Th17 cells can be correlated with disturbances in pregnancy such as spontaneous abortion and pre-eclampsia. We have previously shown that an increase in Th17 cells is accompanied by a decrease in Tregs, and disruption of Treg regulation on Th17 cells occurs in patients with unexplained recurrent spontaneous miscarriage (RSM) (Wang et al., 2010a, b). These findings led us to hypothesize that Th17 cell-derived IL-17 contributes to the initiation of maternal–fetal immunological rejection, and transfusion of Tregs may regulate maternal–fetal immune tolerance and reverse the increased abortion rate caused by IL-17. In this study, we investigate whether intravaginal recombinant IL (rIL)-17 can induce spontaneous abortion in a CBA/J × BALB/c mouse model of normal pregnancy and whether fetal rejection can be prevented by adoptive transfer of pregnancy-induced Tregs from normal pregnant CBA/J mice.

Material and Methods

Experimental animals and grouping

Eight to 10-week-old female CBA/J (h-2k) and male BALB/c (h-2d) mice weighing 20–30 g were obtained from the Beijing Institute of Laboratory Animals of the Chinese Academy of Medical Sciences. The mice were maintained under specific pathogen-free conditions in an animal facility with controlled humidity (55 ± 5%), light (12/12 h light/dark) and temperature (22°C ± 1°C). The air in the facility was passed through an HEPA filter system designed to exclude bacteria and viruses. Animals were fed mouse chow and tap water ad libitum. The experimental protocols and animal care procedures were approved by the Ethics Review Board of Yantai Yuhuading Hospital.

Male BALB/c mice were bred with female CBA/J mice and the vaginal plugs in individual mated female mice were examined daily to determine potential pregnancy. Murine rIL-17 (R&D Systems, Emeryville, CA, USA) was resuspended in sterile phosphate-buffered solution (PBS) at a final concentration of 200 µg/ml. Fifty microliters of PBS (Group 1, n = 20) or rIL-17 (10 µg/mouse) supernatant (Group 2, n = 20) was administered in the vaginal vaults of anesthetized pregnant CBA/J mice by inserting a 100 µl pipette on Day 1 of pregnancy. Twenty-four hours later, vaginal fluids were harvested and assayed for the expression of IL-17 using the enzyme-linked immunosorbent assay as described by Pietrella et al. (2011) (n = 10 from Group 1 or 2). All samples were measured in duplicate. Tregs (2 × 10^7 cells) purified from pregnant CBA/J × BALB/c mice (n = 15) were given intravenously through the tail vein 2 days before mating (Group 3, n = 10) or on Day 7 of pregnancy (Group 4, n = 10).

On Day 14 of pregnancy, the female mice were killed by cervical dislocation, the uteri removed and the implantation sites were documented. The abortion sites were identified by their small size and necrotic, hemorrhagic appearance, compared with normal embryos and placentas. The percentage of abortions was calculated as the ratio of resorption sites to total implantation sites (resorption plus normal implantation sites).

Isolation of Tregs

CD4+CD25+ Tregs were isolated from decidua of normal CBA/J × BALB/c mice on Day 14 of pregnancy using magnetic beads in accordance with the manufacturer’s instructions (MACS, Miltenyi Biotech, Biotech, Germany). The purity of the preparations was between 94 and 97%. The purified Tregs were stimulated with immobilized anti-mouse CD3 antibody (10 µg/ml) and anti-mouse CD28 antibody (10 µg/ml) in the presence of recombinant mouse IL-2 (500 U/ml) in RPMI-1640 with added 1% penicillin/streptomycin, 1% glutamine and 10% heat-inactivated fetal calf serum (eBioscience, San Diego, CA, USA). The cells were exposed to fresh medium every other day. After culture for 3–5 days, Tregs were washed twice with cold PBS, counted, diluted to 2 × 10^7 cells/200 µl PBS and injected i.v. into mice in Groups 3 and 4.

Real-time RT–PCR

Total RNA from decidual tissues was extracted with TRizol (Tianwei, Beijing, China) following the manufacturer’s recommendations, and cDNAs were synthesized according to the instructions provided using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). Primers were designed using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) and each primer was submitted into an NCBI BLAST search to ensure specificity for the target mRNA (Table I). The primers were synthesized by BioAsia Co (Shanghai, China).

Real-time RT–PCR was performed under conditions of 2 min at 95°C, followed by 15 s at 95°C and 50 s at 65°C for 40 cycles. Data were analyzed...
**Western blotting**

Decidual tissues were isolated and washed in cold PBS, pH 7–4, then the tissues were minced in lysis buffer (7 M urea, 2 M thiourea, 30 mM Tris, pH 8.5) containing 50 mM dithiothreitol and protease inhibitors at 4 °C for 20 min by applying gentle pressure. The homogenate was then centrifuged at 12,000 g for 20 min and the collected supernatants were precipitated by ice-cold acetone, stored for 1 h at −20 °C and centrifuged at 20,000 g for 1 h at 4 °C. Precipitates were washed with 90% ice-cold acetone, dissolved in lysis solution and protein concentrations were determined with the Bradford assay (Bio-Rad). Samples separated by sodium dodecyl sulfate polyacrylamide gel (12% w/v) electrophoresis and transferred to polyvinylidene difluoride membranes, blocked with 2% (w/v) skimmed milk for 1 h, then incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-immunoglobulin G at a final dilution of 1: 5000 in blocking solution. Immune-reactive complexes were visualized using a dianinobenzidene kit (Zhong-Shan Biotechnology, Beijing, China). Western blot images were analyzed by densitometric scanning and quantified using ImageQuant TL 7.0 software (GE Healthcare, USA).

**Statistical analysis**

Data are expressed as mean ± SEM, and the differences among the groups were analyzed by the ANOVA or Fisher’s exact test using SigmaStat 3.5. A value of P < 0.05 was considered statistically significant.

**Table I** Primer sequences for RT–PCR using decidua from CBA/J × BALB/c mice.

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<tr>
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</tr>
<tr>
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</tr>
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<td>GGTCTGATCGTAGGATGTT</td>
</tr>
<tr>
<td>IL-10</td>
<td>GAAGACCCCTAGAGGCGG</td>
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IFN-γ, interferon-γ; IL-4, interleukin-4; TGF-β, transforming growth factor-β; IL-10, interleukin-10.

**Table II** Comparison of fetal resorption rates in pregnant CBA/J mice.

<table>
<thead>
<tr>
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PBS, phosphate-buffered saline; Tregs, regulatory T cells.

Fisher’s exact test, *P < 0.05 versus Group 2.

*Group 1, CBA/J × BALB/c received transvaginal PBS; Group 2, CBA/J × BALB/c received transvaginal recombinant IL-17; Group 3, Group 2 + Tregs 2 days before mating; Group 4, Group 2 + Tregs (Day 7 of pregnancy).

**Results**

rIL-17 significantly increases abortion rates in a CBA/J × BALB/c mouse model

The IL-17 concentration in murine vaginal washes of mice injected with PBS (4.6 ± 2.7 pg/ml) was significantly lower than the rIL-17 group (80.5 ± 35.7 pg/ml) (P < 0.05). Normal pregnant CBA/J mice mated with BALB/c males which received transvaginal rIL-17 presented with a significantly increased abortion rate compared with the group which received PBS (27.7 versus 9.9%; P < 0.05). The transfusion of pregnancy-induced Tregs from 14-day normal pregnant mice before mating diminished the abortion rate caused by IL-17 in the CBA × BALB/c mouse model (12.5 versus 27.7%; P < 0.05), while transfusion of Tregs on Day 7 of pregnancy had no effect. Abortion and implantation rates from all groups are shown in Table II.

Expression of IFN-γ in mouse decidua

After confirming the induction of fetal loss by transvaginal rIL-17, we investigated the possible mechanisms involved. We focused on the expression of cytokines secreted by Th1, Th2 and Tregs in the decidua of different groups. Administration of rIL-17 did not affect IFN-γ expression at either the mRNA or protein level (P > 0.05). Moreover, transfusion of Tregs had no effect on IFN-γ mRNA or protein expression (Fig. 1). Representative staining of a western blot, showing all proteins studied, is shown in Supplementary data, Fig. S1.

Expression of IL-4 in mouse decidua

IL-4 is mainly expressed by Th2 cells. The level of IL-4 mRNA appeared to be down-regulated in the group receiving rIL-17 compared with the PBS

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Expression of IL-4 in mouse decidua

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Expression of IL-10 in mouse decidua

The expression of IL-10 mRNA was significantly down-regulated in the group receiving rIL-17 compared with the PBS group (P < 0.05). Transfusion of Tregs increased the IL-10 mRNA level, but this was only significant before mating (P < 0.05; Fig. 4A). The levels of IL-10 protein correlated with that of IL-10 mRNA in each group (Fig. 4B).

Discussion

In normal pregnancy, tolerance of the genetically incompatible fetus by the maternal immune system depends on the interactions of an array
of cytokines secreted by maternal and fetal cells at the site of implantation. Changes in the balance of Th1, Th2, Th17 and Treg cell cytokine profiles may contribute to the failure of implantation of the embryo, development of the placenta and survival of the fetus to term.

The signature cytokines of the Th1, Th2 and Th17 cells subsets are IFN-γ, IL-4 and IL-17, respectively (Saito et al., 2011). Th1 and Th2 cells inhibit the one another’s development through the action of their lineage-specific cytokines IFN-γ and IL-4, respectively. Th2-type cytokines predominate locally in the regulation of the maternal immune response during murine pregnancy (Lin et al., 1993). Increased IFN-γ and decreased production of IL-4 by the decidual T cells has been reported in women with unexplained RSM in comparison with women with normal pregnancy (Raghupathy, 1997). IFN-γ and IL-4 may antagonize initial Th17 development. However, once fully matured, Th17 cells are resistant to the suppressive effects of IFN-γ and IL-4 in vitro. IFN-γ conditioning enhances the capacity of antigen-presenting cells to drive the expansion of memory Th17 cells, and the early Th17 response can induce an optimal Th1 response, facilitate the recruitment of Th1 cells to sites of infection and promote the development of Th1 memory (Lee et al., 2011). We have previously shown that IL-17 is...
highly expressed in the decidua and peripheral blood of patients with unexplained RSM (Wang et al., 2010a,b). In this study, using a murine pregnancy model, we found that transvaginal injection of rIL-17 had no obvious effects on the expression of IFN-γ and IL-4, suggesting that overexpression of IL-17 in the decidua alone may induce inflammation resulting in abortion.

The activity of Th1, Th2 and Th17 cells is attenuated by the anti-inflammatory action of Tregs via the expression of TGF-β, IL-10, cytotoxic T-lymphocyte antigen 4 (CTLA-4) and other factors (Saito et al., 2013). Tregs are often classified as one of two subpopulations, the naturally occurring regulatory T cells (nTregs) producing TGF-β, and induced regulatory T cells (iTregs) producing IL-10, which arise in the periphery from naive T cells upon exposure to TGF-β. The depletion of Tregs induced abortion in allogeneic pregnancy in mice, and a decreased level of Tregs in the cells upon exposure to TGF-β were involved in Tregs expansion and recruitment of Tregs from peripheral blood to the decidua (Tilburgs et al., 2008; Robertson et al., 2010a,b). In the present study, transfusion with Tregs prior to mating antagonized the common requirement for the cytokine TGF-β. In response to TGF-β in vitro as well as in vivo, many T cells coexpress RORγt and Foxp3 (Bettelli et al., 2006). TGF-β has been shown to maintain peripheral nTregs that develop in the thymus and to induce the differentiation of naive CD4+ T cells to iTregs. Interestingly, TGF-β is also important in Th17 differentiation. In the presence of TGF-β and IL-6, naive CD4+ T cells differentiate into Th17 cells. Transvaginal TGF-β promotes a regulatory T-cell response enhancing the success of pregnancy in an established model of abortion (Yang et al., 2008; Clark et al., 2008; Sunder and Lenton, 2000). In our study, transvaginal injection of rIL-17 decreased the expression of IL-10 and TGF-β, and transfusion of Tregs prior to mating antagonized the response to rIL-17.

Paternal antigens, human pregnancy hormones such as hCG, and chemokines were involved in Tregs expansion and recruitment of Tregs from peripheral blood to the decidua (Tilburgs et al., 2008; Robertson et al., 2009; Schumacher et al., 2009). In a murine model of disturbed fetal tolerance, a reduced frequency of systemic and local Tregs was clearly associated with fetal resorption. Adoptive transfer of Tregs into abortion-prone females rescued those females from abortion (Zenciusen et al., 2005; Yin et al., 2012). We have previously shown that IL-17 expression in stimulated CD4+ T cells can be inhibited by Tregs, and this regulation was disturbed in patients with unexplained RSM. Cell-to-cell contact, TGF-β and IL-10 are necessary for this suppression (Wang et al., 2010a,b). In the present study, transfusion with Tregs expanded in vitro from normally pregnant mice prior to mating significantly increased IL-10 and TGF-β in decidua and lowered the fetal resorption rates. However, Treg transfer failed to prevent abortion if carried out on Day 7 of pregnancy, suggesting that Tregs are important in the implantation phase and early stages of pregnancy, but may not be necessary for maintenance of the later stages of pregnancy (Shima et al., 2010). These research findings have potential applications in the prevention of abortion, based on the transfer of antigen-specific Tregs before mating takes place.

### Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

### Authors' roles

W.W.J was responsible for the experiment design and manuscript drafting. L.F.J and L.X were responsible for the data acquisition and analysis. H.C.F., B.H.C., Q.Q.L. and L.X.M were responsible for the data interpretation and critical discussion.

### Funding

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### Conflict of interest

None declared.

### References


Piccinni MP, Beloni L, Livi C, Maggi E, Scarselli G, Romagnani S. Defective production of both, leukemia inhibitor factor and type 2 T-helper...


