The deregulation of regulatory T cells on interleukin-17-producing T helper cells in patients with unexplained early recurrent miscarriage

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BACKGROUND: CD4+CD25+ regulatory T cells (Tregs) are important for the maintenance of immune homeostasis by virtue of their ability to control T-cell proliferation in the peripheral blood (PB). We recently demonstrated that the prevalence of Tregs is decreased, whereas that of Th17 cells is increased, in the PB and decidua samples of patients with unexplained recurrent miscarriage (RM). In this study, we investigated whether the cytokine production of Th17 cells can be suppressed by the Tregs and elucidated the mechanism by which Tregs exert this suppressive effect.

METHODS: Flow cytometry was used to analyze the surface phenotype and cytokine production of Th17 cells in the PB of women with unexplained RM (n = 17) and healthy women in early stages of pregnancy who underwent elective abortion (n = 20). The suppressive ability of Tregs on Th17 cells was assessed in vitro co-cultures and transwell experiments. The amount of secreted interleukin-17 (IL-17) in the supernatants was measured by enzyme-linked immunosorbent assay (ELISA). The inhibitory activity of transforming growth factor-β (TGF-β) and IL-10 on IL-17 expression in CD4+ T cells was assessed using ELISA.

RESULTS: The proportions of IL-17-positive CD4+ T cells, CC chemokine receptor type 6 (CCR6)-positive CD4+ T cells and CCR6 expression of IL-17-positive CD4+ T cells were higher in the PB samples of patients with unexplained RM than in PB of healthy control subjects. In vitro, Tregs could inhibit the expression of IL-17; more Th17 cells were inhibited in the control group than in the unexplained RM group. High-dose TGF-β inhibited the expression of IL-17, whereas IL-10 inhibited IL-17 expression in a dose-dependent manner.

CONCLUSIONS: IL-17 expression can be inhibited by Tregs. The suppressive activity of Tregs on Th17 cells was decreased in patients with unexplained RM. The ability of Tregs to suppress cytokine secretion might be effected by a cell–cell contact. TGF-β and IL-10 could inhibit the expression of IL-17.

Key words: recurrent abortion / regulatory T cells / Th17 cells / transforming growth factor-β / interleukin-10

Introduction

Unexplained recurrent miscarriage (RM) is defined as the loss of three or more consecutive pregnancies before the 20th week of gestation despite the normal findings in routine screening tests (normal appearance of the uterine cavity; normal parental karyotypes and normal endocrinal, infectious and autoimmune parameters). This condition is thought to be caused by the allorejection of the fetus by the mother.

CD4+CD25+ regulatory T cells (Tregs) express the forkhead transcription factor Foxp3 and participate in the maintenance of immunological self-tolerance by actively suppressing self-reactive lymphocytes via mechanisms that are mediated by a cell–cell contact and production of soluble factors [transforming growth factor-β (TGF-β) and interleukin (IL)-10; Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003]. Tregs can also suppress the maternal alloresponses targeted against the fetus. Defects in the numbers or functions of Tregs have been documented in cases of miscarriage and unexplained RM (Sasaki et al., 2004; Zencussen et al., 2005; Yang et al., 2008a,b). The newly identified subpopulation of CD4+ effector T cells, Th17, is distinct from the well-described Th1, Th2 and Tregs.
populations (Harrington et al., 2005; Park et al., 2005). Th17 cells are regulated by the orphan nuclear receptor, retinoic acid-related orphan receptor-γt (RORγt); secrete their signature cytokine, IL-17; and preferentially express the chemokine receptors, CC chemokine receptor type 6 (CCR6; Acosta-Rodriguez et al., 2007).

Th17 cells share a complex relationship with Tregs and may thereby affect Treg-induced transplant tolerance. In our previous study, we confirmed the prevalence of Th17 cells and the inverse relationship between the proportions of Th17 cells and Tregs among the peripheral blood lymphocytes (PBLs) and decidua samples of women with unexplained RM (Wang et al., 2010). This study is the first to report the deregulation of Th17 cells by Tregs in unexplained RM.

Materials and Methods

Subjects

All subjects were managed by the outpatient department of the Gynecology Specialist Clinic at Renji Hospital (Shanghai, China) between August 2008 and October 2009. In this study, we included 17 women with unexplained RM and 20 healthy women at early stages of pregnancy and obtained blood samples from them. The diagnosis of unexplained RM was made after excluding verifiable causes, such as abnormalities of the uterus or cervix, chromosomal abnormality, infection (chlamydia, ureaplasma and TORCH syndrome), endocervical disorders (luteal function defect, hyperprolactinemia, polycystic ovary syndrome and hyperandrogenemia), metabolic diseases (diabetes, insulin resistance, hyperthyroidism and hypothyroidism), congenital thrombophilies and autoimmune diseases. To exclude antiphospholipid syndrome, the subjects were tested for lupus anticoagulation antibodies, antcardiolipin antibodies and anti–beta2-GPI antibodies on at least five occasions, with 6-week intervals (Bao et al., 2008). Male partners of all these patients had normal semen status, which was defined according to the criteria proposed by the World Health Organization (1999). For patients with unexplained RM, the urine HCG was measured on the 30th day of the menstrual cycle. If the urine HCG was positive, then serum β-HCG levels were tested two times every other day to determine pregnancy. Fetal heart activity was assessed using Doppler ultrasonography with a 6-MHz transducer, at 7 and 9 weeks of gestation. When no or abnormal fetal heart activity was found, the patients were advised to undergo tests for serum β-HCG levels, with additional ultrasonography examinations and serum β-HCG tests performed every other week. The patients were advised to undergo an induced abortion when the fetal heartbeat was not detected in any of the examinations or when it disappeared after being detected previously.

The mean age of patients with unexplained RM was 31.9 ± 4.8 (range: 25–40) years. They had a mean of 3.7 ± 1.2 (range: 3–6) miscarriages, at an average of 66.5 ± 12.9 days of gestation. The mean age of the women in the control group was 27.6 ± 6.2 (range: 26–36) years, and all these women had one living child and no history of spontaneous abortion, ectopic pregnancy or preterm delivery. Further, in all women of the control group, fetal heart activities were identified during the week before the abortion, and elective terminations were conducted on average at 69.2 ± 11.8 days of gestation. This study was approved by the ethics review board of Renji Hospital, and written consent was obtained from each participant.

Flow cytometry

For the analysis of Th17 cells, 2 × 10⁶ PBLs were stimulated for 4 h with 50 ng/ml of phorbol myristate acetate (PMA) and 1 μM ionomycin in the presence of 10 μg/ml of brefeldin A (Alexis Biochemicals, San Diego, CA, USA) in 24-well plates. Upon harvest, cells were aliquoted into 5 ml sterile tubes, washed once in phosphate-buffered saline and stained for the surface marker by incubation with FITC-conjugated antihuman CD4 and APC-conjugated antihuman CCR6 at 4°C for 30 min (BD Biosciences, San Jose, CA, USA). Subsequently, the cells were stained with PE-conjugated antihuman IL-17A (eBioscience San Diego, CA, USA) for Th17 cell detection after fixation and permeabilization according to the manufacturer’s instructions. Samples were analyzed using a fluorescence-activated cell sorting (FACS) Calibur obtained from BD Biosciences.

Extraction and purity of PBLs

PBLs were isolated by the standard Ficol–Hypaque density centrifugation. CD4⁺ T cells were purified using MACS columns for positive selection. The purity of the CD4⁺ T cells purified by this method was up to 95%, as confirmed by flow cytometric analysis. Purified CD4⁺ T cells were then incubated with anti-CD25 mouse monoclonal antibodies, and CD4⁺CD25⁺ T cells were separated from CD4⁺CD25⁻ T cells. The purity of CD4⁺CD25⁺ T cells was ~90%. The lymphocytes were cultured in the RPMI-1640 medium, supplemented with 1% penicillin/streptomycin, 1% glutamine and 10% heat-inactivated fetal calf serum (Gibco BRL, Invitrogen Corporation, USA).

Treg cells in vitro suppression assays

For direct suppression assays, 5 × 10⁴ CD4⁺ T cells, CD4⁺CD25⁻ Treg cells and CD4⁺CD25⁺ T cells were cultured in 24-well plates coated with 10 μg/ml of mouse antihuman CD3 antibodies and 10 μg/ml of mouse antihuman CD28 antibodies (Gibco, Invitrogen). The cell culture medium contained RPMI-1640 with added 1% penicillin/streptomycin, 1% glutamine and 10% heat-inactivated fetal calf serum. After incubation for 6 days at 37°C in 5% CO₂, cell-free supernatants were collected, the concentrations of IL-17 in supernatants were assessed using an enzyme-linked immunosorbent assay (ELISA) kit (Biosource, Nivelles, Belgium) and the expressions of IL-17 and CCR6 were measured by FACS.

Transwell experiments

CD4⁺CD25⁺ T cells were co-cultured with CD4⁺CD25⁻ T cells using a semi-permeable transwell membrane (Nunc, Denmark). CD4⁺CD25⁺ T cells (5 × 10⁴) isolated from the blood samples of patients with unexplained RM or control women were added to the upper chambers of the transwells, whereas isolated CD4⁺CD25⁻ T cells (5 × 10⁴) were added to the respective lower chambers in an autologous setting. After incubation for 6 days at 37°C in 5% CO₂, the cells were analyzed for expression of IL-17 and CCR6, as described above.

TGF-β or IL-10 suppression assays

For TGF-β or IL-10 suppression assays, purified CD4⁺ T cells (5 × 10⁴) were isolated from the PB samples of patients with unexplained RM; these cells were then stimulated with T–cell activation beads (antihuman CD3/CD28 antibodies) and different concentrations of TGF-β or IL-10 (0, 0.1, 1, 5 and 10 ng/ml) in the presence of a medium (R&D Systems, USA). After being cultured for 6 days, the cells were analyzed for IL-17 expression.

Analysis

Data are presented as the mean ± SEM. Statistical analyses were performed using SPSS statistical software (version 11.01; SPSS, Chicago, IL, USA). The Student’s t-test was used for comparing the two independent groups. P-values of <0.05 were considered significant.
**Results**

**Increased frequency of IL-17^+CCR6^+**-positive CD4^+ T cells among PBLs from patients with unexplained RM

Using flow cytometry, we evaluated the expression of IL-17A in the PBLs of the two groups. Consistent with the results of our previous study, the findings in this study indicated a larger proportion of IL-17A-producing CD4^+ T cells among the PBLs of patients with unexplained RM than among the PBLs of healthy control subjects (0.6% ± 0.05 versus 0.3% ± 0.03; *P* = 0.041; Fig. 1A).

We also observed that the expression of CD4^+CCR6^+ T cells in PB in patients with unexplained RM was higher than that in the controls (60.5% ± 7.3 versus 46.2% ± 5.9; *P* = 0.035; Fig. 1B). The analysis of CCR6 expression confirmed that a large proportion of CD4^+ IL-17-producing T cells expressed CCR6 and that the expression of CCR6 on Th17 cells was higher in the samples of patients with unexplained RM than in those of the healthy control subjects (86.3% ± 11.8 versus 70.2% ± 10.3, *P* = 0.021; Fig. 1C).

**CD4^+CD25^+** regulatory T cells have a suppressive effect on CD4^+IL-17 cells

IL-17 concentration in the culture supernatants of the CD4^+ T cells of patients with unexplained RM was significantly higher than that in those of the CD4^+ T cells of healthy control subjects (*P* = 0.017).

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**Figure 1** Enrichment of CD4^+IL-17A^+ T cells, CD4^+CCR6^+ T cells and CD4^+IL-17A^+CCR6^+ T cells in the PB of patients with unexplained RM (URM). Representative images showing the results of FACS staining of PBLs from one of the patients with unexplained RM and healthy women in early stages of pregnancy (control) are shown. (A) Numbers of CD4^+IL-17A^+ expressing cells presented as a percentage of PBLs from unexplained RM and control women. (B) Percentage of CD4^+ T cells expressing CCR6 in the PBLs isolated from unexplained RM and control women. (C) Proportion of cells expressing CCR6 among CD4^+IL-17A^+ T cells in PBLs isolated from patients with unexplained RM (*n* = 17) and control (*n* = 20) women. (D) Bars represent the median (B and C). Values are expressed as the mean and SEM.
There was no significant difference in the IL-17 concentrations in the supernatants of CD4^+CD25^- T cells of patients with unexplained RM and those of the healthy controls (P = 0.258; Fig. 2A). IL-17 was not detected in CD4^+CD25^+ T cell culture supernatants.

When CD4^+CD25^+ T cells were separated from CD4^+ T cells, the concentration of IL-17 in the CD4^+CD25^- T cell population was found to be significantly higher than that in the CD4^+ T cells in both the unexplained RM group and the control group (P = 0.001 and P = 0.036, respectively; Fig. 2A).

When a cell–cell contact between PB CD4^+CD25^+ T cells and CD4^+CD25^- T cells (1:1) was prevented by a semi-permeable transwell membrane, the suppressive effects of CD4^+CD25^+ T cells were abrogated in the unexplained RM group (P = 0.060) but not in the control group (P = 0.020; Fig. 2A); however, there was no difference between the IL-17 levels in these two groups (P = 0.085). The intracellular expression of IL-17 was consistent with its extracellular expression (Fig. 2B). The CCR6 expression levels did not differ when cultured with three different measures (data not showed).

We used ELISA to examine the in vitro effects of TGF-β and IL-10 on IL-17 production by Th17 cells. Because TGF-β is central to the development and function of Th17 cells, we first examined the potential role of TGF-β in IL-17 production by using CD4^+ T cells isolated from patients with unexplained RM. We found that the expression of IL-17 in CD4^+ T cells was not influenced by the presence of TGF-β at concentrations of 0.1–5 ng/ml, but the production of IL-17 in the culture fluid of CD4^+ T cells decreased significantly in the presence of TGF-β at a concentration of 10 ng/ml (Fig. 3A).

In vitro culture of CD4^+ T cells resulted in a significant decrease in the expression of IL-17 at the concentrations of 1–5 ng/ml of IL-10. IL-10 induced a decrease in the expression of IL-17 in a dose-dependent manner (Fig. 3B), a finding similar to that reported by Heo et al. (2010).

**Discussion**

The discovery of the Treg/Th17 balance significantly challenged the Th1/Th2 dichotomy model proposed two decades ago (Mosmann et al., 1986).
and Coffman, 1989). Tregs and Th17 cells are two lymphocyte subsets with opposing actions. In normal pregnancy, Tregs prevent the generation of an immune response against fetal tissue and a decrease in the number of Tregs is associated with abortion. In contrast to the Tregs, Th17 cells promote inflammatory, autoimmunity and transplant rejection in humans and increased Th17 cells accompanied with decreased Tregs had been shown in unexplained RM patients (Wang et al., 2010).

The functions of Tregs are mainly mediated by a cell–cell contact or by the production of anti-inflammatory cytokines such as IL-10 and TGF-β, whereas Th17 cells mainly exert their effects by secreting IL-17. IL-17 has pleiotropic activities, including induction of the expression of proinflammatory cytokines and chemokines, which mediate tissue infiltration and destruction.

Differential expressions of chemokine receptors direct the migration of leukocytes into distinct tissues and microenvironments. The majority of human PB Th17 cells express CCR6, and the migration of Th17 in vitro is regulated in a CCR6-dependent manner. Loss of CCR6 expression in Th17 cells decreases the severity of experimental autoimmune disease and Th17 cells recruitment into inflammatory tissues (Hirota et al., 2007). In this study, we demonstrated that the numbers of CD4+ T cells that produce IL-17 were increased significantly in the PB of patients with unexplained RM, which suggests that Th17 may play a role in unexplained RM. In accordance with this, increased expression of CCR6 was found in both CD4+ T cells and CD4+IL-17A+ cells in the PB of patients with unexplained RM.

During pregnancy, isolated CD4+CD25+ T cells suppress the proliferative responses of autologous CD4+CD25− T cells (Somerset et al., 2004; Zenzlussen et al., 2005). Some reports have been published on whether Tregs regulate the pathogenic function of Th17 cells. Annunziato et al. (2007) showed that in Crohn’s disease, Tregs can suppress the proliferation of Th1 and Th2 clones but not Th17 cells. Similarly, IL-17 secretion could not be suppressed by Foxp3+ Tregs isolated from healthy controls (Evans et al., 2007). In contrast, Cao et al. (2004) showed that Tregs isolated from the joints of rheumatoid arthritis patients could inhibit the secretion of IL-17 from effector T cells. Proliferation of T cells that differentiated in vitro in the presence of TGF-β and IL-6 could be suppressed both by naturally occurring CD4+CD25+ Tregs and by in vitro TGF-β-converted Foxp3+ T cells (Bettelli et al., 2006). Tregs-dependent control of Th17 responses is essential for the maintenance of immune homeostasis (Chaudhry et al., 2009). In this study, the results suggest that CD4+CD25+ Tregs have a suppressive potential on IL-17 expression in CD4+ T cells and that a cell–cell contact is necessary for the former cells to exert their inhibitory effects. Besides cell–cell contact, other mechanisms may be involved in the regulation of IL-17 expression. The inhibitory effect of CD4+CD25+ T cells on IL-17 expression was reduced in unexplained recurrent spontaneous miscarriage.

We wondered whether cytokines can regulate IL-17 expression in patients with unexplained RM who overexpress IL-17. TGF-β is the major cytokine produced by Tregs that exert inhibitory effects, and it regulates the differentiation of Tregs as well as Th17 cells (Bettelli et al., 2006; Mangan et al., 2006). TGF-β appears to have multiple effects on human Th17 cells. TGF-β activates the transcription factor STAT3 (signal transducer and activator of transcription 3) and the expression of another transcription factor, RORγt, thereby leading to the differentiation and development of Th17 cells (Takatori et al., 2008; Volpe et al., 2008; Yang et al., 2008a,b). Das et al. (2009) reported that TGF-β does not directly promote Th17 cell differentiation; instead, it acts indirectly by blocking the expression of the transcription factors, STAT4 and GATA-3, thus preventing Th1 and Th2 cell differentiation. Evans et al. (2007) reported that TGF-β actually inhibited IL-17 expression. In the present study, we found that TGF-β had no effect on Th17 cells in low doses, but suppressed IL-17 expression at high doses.

IL-10, a 37-kDa homodimer, is another cytokine that mediates the inhibitory effects of Tregs. IL-10 exerts its effects by signaling through the IL-10 receptor, negatively regulates the expression of Th17 cytokines and RORγt, decreases the amount of IL-17 and plays a role in preventing exaggerated inflammatory and immune responses (Gu et al., 2008). In this study, we found that when CD4+ T cells were cultured in the presence of IL-10, IL-17 expression decreased significantly as the concentration of IL-10 increased. This suggests that IL-10 could be an attractive therapeutic target.

In conclusion, we found that the IL-17 expression levels in the PB of patients with unexplained early RM were elevated and that CCR6 expression was increased in IL-17+CD4+ T cells in the embryonic period. This period comprises the first 8 weeks of development of the conceptus after fertilization. Tregs can reduce the IL-17 production but the suppressive ability was decreased in unexplained early RM; this finding suggested Tregs have the ability to maintain ‘tolerant maternal-fetal microenvironment’ during early pregnancy and that the decreased suppressive ability of Tregs may play a role in the pathogenesis of unexplained RM. We expect that treatment measures directed at achieving a balance between Th17 cells and Tregs by altering the TGF-β and/or IL-10 levels may be a promising option for unexplained RM.

Authors’ roles
W.-J.W. designed the study, analyzed the results and drafted the manuscript. C.-F.H., Q.-L.Q. and X.W. helped with the clinical evaluation of the RM patients. L.-H.Q. collected blood samples. Q.-D.L. provided intellectual input and helped in finalizing the manuscript.

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