Original Article
Decidual and peripheral blood TCRαβ⁺CD3⁺CD4⁻CD8⁻ double negative regulatory T cells in early pregnancy subjects and unexplained recurrent spontaneous abortion patients

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Abstract: Accumulating evidences have demonstrated that TCRαβ⁺CD3⁺CD4⁺CD8⁺ NK1.1-double negative regulatory T cells (DN Tregs) are highly potent immune suppressors and play an important role in down regulating immune responses in autoimmunity, transplant rejection, graft-versus-host disease (GvHD). Whether human DN Tregs play a role in maternal-fetal immunological tolerance hasn’t been reported yet. In the present study, to explore the possible role of human DN Tregs in unexplained recurrent spontaneous abortion (URSA), we compare the frequency and function of peripheral and/or decidual DN Tregs of 22 women with normal history of reproduction, 31 normal early pregnant women and 42 URSA patients through flow Cytometry and cell proliferation assays. The proportion of DN Tregs in decidua was significantly higher than peripheral blood both in URSA patients and normal early pregnant women. It is the proportion but not the suppressive ability of DN Tregs decreased in peripheral blood and decidua of URSA patients than normal early pregnant women. Our result suggests that human DN Tregs enrich in decidua and may contribute to the mechanisms mediating maternal-fetal immunological tolerance and may be involved in protection the fetus during pregnancy in human. Also, DN Tregs may serve as a novel target in URSA treatment.

Keywords: Decidua, DN regulatory T cells, pregnancy, recurrent, spontaneous abortion

Introduction

Recurrent spontaneous abortion (RSA), two or more consecutive pregnancy losses prior to 20 weeks, occurs in about 1% of all pregnancies [1]. Except for chromosomal, anatomic, endocrinologic, infectious and auto-immunologic abnormalities, there are 50% of RSA cases remain unexplained. The breakage of maternal-fetal immunologic tolerance have been implicated in the pathophysiology of unexplained recurrent spontaneous abortion (URSA) [2].

The mechanisms preventing the maternal immune system to reject its semi-allogeneic fetus are still poorly understood. Medawar first proposed the concept that human pregnancy is similar to a successful semi-allograft implantation and pregnancy being a state of immunological tolerance [3]. Localized mechanisms such as the expression of HLA-G may play an important role in fetal evasion of maternal immune attack [4]. Further, Th1/Th2 cytokine balance has been seen as another very important mechanism determining the survival of the fetus in the maternal uterus [5]. In 2004, Aluvihare reported that CD4⁺CD25⁺Forkhead box P3 (Foxy3)⁺ Treg cells might mediate maternal tolerance to the fetus in mice [6]. Also, it has been demonstrated that adoptive transfer of Treg cells derived from normal pregnant mice could prevent fetal rejection in vivo, but transfer of Treg cells from nonpregnant mice was ineffective [7]. In humans, previous research show that CD4⁺CD25⁺Foxy3⁺ Treg cells accumulate in the decidua in normal pregnancy, and the population of CD4⁺CD25⁺Foxy3⁺ Treg cells is decreased in miscarriage [8-10]. The suppres-
sive activity of Tregs decreased in women with unexplained recurrent miscarriage [11].

Recently, a novel subset of TCRαβ⁺CD3⁺CD4⁻CD8⁻ double negative regulatory T cells (DN Tregs) has been described. It comprises 1-3% of peripheral T cells in normal mice and humans [12, 13]. It had been demonstrated that both rodent [14, 15] and human DN Tregs [16] lack expression of Foxp3. In murine, DN Tregs can suppress Ag-specific auto-, allo- or xeno-reactive CD8⁺ [12, 17] T cells, CD4⁺ [18] T cells, B cells [15, 19] or NK cells [20] and dendritic cells [21]. In mice, DN Tregs dominate female genital tract [22]. It plays an important role in preventing allograft rejection [18], graft-versus-host disease (GvHD) [23, 24] and autoimmune diabetes [15, 17]. In human, a remarkable increase has been made in the knowledge of the mechanisms of its function in recent years. In contrast to CD4⁺Foxp3⁺ Tregs, human DN Tregs have to be activated by allogeneic APCs or beads coated with anti-CD3 and anti-CD28 antibodies to induce their regulatory potential. Interestingly, human DN Tregs suppress proliferation of responder T cells by cell contact-dependent mechanisms and the suppression were reversible [16].

Whether human DN Tregs contribute to the maintaining of maternal-fetal tolerance has not been reported yet. Furthermore, current studies of DN Tregs in disease models are largely based on murine models. For human being, few researches have been done. In the present study, we compared the proportion of peripheral and/or decidual DN Tregs in the setting of women with normal history of reproduction, normal early pregnancy women and URSA patients. In addition, we performed functional analysis on peripheral and decidual DN Tregs to evaluate their anti-proliferation effects on activated CD4⁺ and CD8⁺ autologous T cells.

### Materials and methods

#### Subjects

All the subjects enrolled were outpatients at the Department of Gynecology or Physical Examination Center, Provincial Hospital affiliated to Anhui Medical University, Hefei, China. 42 patients who had at least three consecutive spontaneous early miscarriages of unexplained etiology with the same partner were recruited to participate in the study. The diagnosis of “unexplained” miscarriage was made as the one defined by the Practice Committee of the American Society for Reproductive Medicine [1]. No URSA subjects had risk factors such as uterine malformation, chromosomal abnormality, infection (chlamydia, ureaplasma and TORCH syndrome), endocrinial disorders (luteal function defect, hyperprolactinemia, polycystic ovary syndrome and hyperandrogenemia), metabolic diseases (diabetes, insulin resistance, hyperthyroidism and hypothyroidism), maternal thrombophilias and antiphospholipid syndrome. All recruited URSA abortuses had been karyotyped, to exclude any chromosome problems. At the same time, 31 healthy early pregnant women who were undergoing elective termination during the first trimester and 22 non-pregnant healthy women with normal history of reproduction aged between 23 and 35 years old who were undergoing their physical examination were randomly selected in this study. In all 31 normal pregnant cases, fetal heart activity had been identified through B ultrasonography. There were no significant differences in age and pregnancy duration among these three groups (Table 1).

The study was conducted in compliance with the Declaration of Helsinki and was approved by the Ethics Committee of Provincial Hospital affiliated to Anhui Medical University (2011 Ethics 83rd). Signed informed consent was obtained from all women.

#### Sample preparation

Heparinized venous blood was obtained from all of the subjects according to protocols approved by the Institutional Review Board (IRB) of Provincial Hospital affiliated to Anhui Medical University. For the analysis of the proportion of DN Tregs, the peripheral blood samples were used directly, while for the in vitro suppression
assays of DN Tregs, peripheral blood mononuclear cells (PBMCs) were isolated by the standard Ficoll-Hypaque density centrifugation. Decidual samples were obtained from patients with induced abortion, representing early pregnant deciduas, and immediately from patients with spontaneous abortion, representing abortion samples. None of the decidual samples showed any evidence of necrosis or acute inflammation. The decidual mononuclear cells (leukocytes) were purified by the Ficoll-Hypaque method after mechanical disruption and filtration through a 32-μm nylon mesh, as previously reported [25].

Flow cytometry

The peripheral blood sample and decidual leukocytes were stained directly with the following monoclonal antibodies (all purchased from BD Biosciences): TCR-αβ-FITC, CD4-PE, CD8-PerCP-Cy5.5, CD3-APC or isotype IgG as controls. Flow cytometry was performed 6 days later.

CFSE labeling and cell proliferation assays

Fresh isolated PBMCs from the same subject with DN Tregs were resuspended in RPMI 1640, After incubation for 2 h at 37°C in 5% CO₂, non-adherent cells were harvested and washed once and suspended in PBS at a density of 1x10⁶/ml. Equal volume PBS containing 5 µmol/L CFSE was added into the lymphocytes suspension and incubated for 10 min at 37°C. CFSE-labeled lymphocytes were cultured in medium containing RPMI-1640 with added 10% FBS at a density of 1x10⁶/ml.
Figure 1. Flow cytometric analysis of TCR-αβ⁺CD3⁺CD4⁻CD8⁻ DN Tregs in decidua in normal early pregnant individuals and URSA patients. Decidual leukocytes were stained with anti-CD3-APC, anti-CD4-PE, anti-CD8-PerCP-Cy5.5, and anti-TCR-αβ-FITC mAbs and analyzed by flow cytometry. Regions were set on the CD3⁺ T cells based on their forward and side scatter properties and their CD3 expression. The frequency of TCRαβ⁺CD3⁺CD4⁻CD8⁻ DN Tregs was determined as the percentage of TCRαβ⁺CD4⁻CD8⁻ cells on gated CD3⁺ T cell population. Representative results of flow cytometric analysis on decidua leukocytes from a normal early pregnant individual and an URSA patient are presented.
and granulocyte populations. The in vitro proliferation of CD4+ T cells or CD8+ T cells in the presence or absence of DN Tregs was measured by calculating the percentage of CFSE dim cells in CD4+ T cell or CD8+ T cell population.

**Statistical analysis**

Student’s t-test and one-way ANOVA were used for data with homogeneous variance. While Mann Whitney U test and Kruskal-Wallis test were used for heterogeneous variance data. All data analyses were processed by SPSS 15.0 statistical software. P<0.05 was considered significant.

**Results**

*DN Tregs accumulate in the decidua in human in case of pregnancy*

The proportions of DN Tregs in decidua were significantly higher than that in peripheral blood both in URSA patients (1.82±0.85% vs. 0.92±0.68%, P<0.01) and normal early pregnant women (5.26±3.58% vs. 1.56±1.07%, P<0.01) (Table 2). These data indicate that
DN Tregs and URSA

The population of DN Tregs as a percentage of total CD3+ T cells was evaluated by flow cytometric analysis using the gating strategy shown in Figure 1. The findings in this study indicated a decreased proportion of DN Tregs both in the peripheral blood lymphocytes (0.92±0.68% vs. 1.56±1.07%, P<0.05) and in deciduas leukocytes (1.82±0.85% vs. 5.26±3.58%, P<0.05) of patients with URSA than their normal early pregnant counterpart (Table 2).

To investigate the suppressive potential of human decidual and peripheral blood DN Tregs, coculture experiments were performed. CFSE-labeled decidual and peripheral blood lymphocytes were cocultured with anti-CD3/CD28 beads in the presence or absence of allo-activated DN Tregs and proliferation of decidual

**Figure 3.** Decidual DN Tregs are able to suppress proliferation of autologenetic CD4+ and CD8+ T cells in URSA. FACS profiles are from 1 experiment of 3 performed.
and peripheral blood lymphocytes cells was measured by flow cytometry. After 5 days, decidual and peripheral blood lymphocytes cells revealed a strong proliferation, which was significantly suppressed by the addition of DN Tregs (Figures 2 and 3). The suppressive effect of peripheral blood DN Tregs was similar among non-pregnant, normal pregnant and URSA patients. Also, the suppressive effect of decidual DN Tregs in URSA patients was similar with that of normal pregnant women.

Discussion

Suppression of immune responses by regulatory T (Treg) cells is one of the major mechanisms for the induction and maintenance of immuno-tolerance [26, 27]. Tregs have been shown to play an important role in a wide range of immune processes, including autoimmune disease [26, 28], transplantation tolerance [29, 30], GvHD [31] and cancer [32]. It has been confirmed that regulatory T cells consist of many distinct T cell subsets, including CD4+CD25+Foxp3+ T cells, T-regulatory type 1 (Tr1) cells, T-helper 3 (Th3) cells, CD8+CD28- T cells, and TCRαβ+CD3+CD4-CD8- T cells [33, 34]. The group of L. Zhang was the first to identify and characterize TCRαβ+CD3+CD4-CD8-NK1.1-T cells as antigen-specific double negative regulatory T cells (DN Tregs) in vitro and in vivo [12]. Subsequent reports from various laboratories confirmed and extended these findings in different disease models [13, 14, 18, 19]. Our study is the first in exploring the probably role of human DN Tregs in maternal-fetal tolerance.

DN Tregs seem to preferentially dwell in specific organs or tissues [35]. A previous study showed that DN Tregs were the dominant lymphocyte population (70%-90%) in the genital tract of naive, pregnant, or Chlamydia trachomatis-infected C57BL/6 mice [30]. Some previous studies demonstrate that DN Tregs can home into tolerant allografts to suppress immune responses locally and prevent the rejection of donor-specific allografts [36-38]. Our study showed for the first time that either in normal early pregnant woman or for URSA patients, the proportion of DN Treg in decidua was significantly higher than peripheral blood. The molecular mechanism leading to the selective accumulation of DN Tregs to deciduas was unknown. More researches should focus on the identification of molecules that are critical for DN Tregs migration.

In 2000, DN Tregs was first described having the ability in the induction of donor-specific transplantation tolerance to MHC-mismatched allografts [12]. Subsequent studies also showed that DN Tregs could induct transplantation tolerance to skin [39] and islet [14] allografts as well as cardiac xenografts [40] in donor-specific manner. Human pregnancy represents a condition of semiallograft to maternal host because of the presence of paternally derived antigens. However, mechanisms preventing the maternal immune system to reject its semiallogeneic fetus are still poorly understood. URSA is thought to be caused by the allorejection of the fetus by the mother. In this study, we demonstrated for the first time that the proportion of DN Tregs both in peripheral blood and in decidua decreased in URSA patients than normal early pregnant women. Moreover, in decidual, the significance was more obvious. The results suggest that the decreased proportion of DN Tregs in peripheral blood and decidua may play an important role in the pathogenesis of URSA in human.

In this study we have examined the suppressive function of human DN Tregs in non-pregnant woman, normal early pregnant woman and URSA patients. We demonstrate that human DN Tregs are highly potent suppressor cells of CD4+ and CD8+ T cell responses in these patients. However, our data reveal that the suppressive effects of DN Tregs in peripheral blood and decidua were similar among these three groups.

Conclusions

In summary, our data show that human DN Tregs enrich in decidua during pregnancy. Its proportion decreased in women with URSA, although it suppressive capacity did not change. The results suggest that DN Tregs may contribute to the mechanisms mediating maternal-fetal immunological tolerance and may be involved in protection the fetus during pregnancy in human. It also suggests that DN Tregs may serve as a novel target in URSA treatment.

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Disclosure of conflict of interest

None.

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