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Effects of estrogen on $CD4^+$ $CD25^+$ regulatory T cell in peripheral blood during pregnancy

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ABSTRACT

Objective: To investigate the effects of estrogen (E_2) level on regulatory T cells (Treg) in peripheral blood during pregnancy. **Methods:** A total of 30 healthy non-pregnant women were selected as control group, 90 pregnant women of early, middle and late pregnancy and 30 postpartum women at 1 month after parturition were selected as experimental groups including early pregnancy group, middle pregnancy group and late pregnancy group; the proportions of $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^+$ $CD127^-$ Treg among $CD4^+$ T cells were detected by flow cytometry; the serum estrogen content in peripheral blood was detected by electrochemical immune luminescence method. **Results:** E_2 level was coincident with the change of Tregs number during pregnancy. The estrogen content in peripheral blood increased gradually from early pregnancy to late pregnancy, then decreased significantly after parturition, and the level at 1 month after parturition down to the level in non- pregnancy group ($P>0.05$); the level of E_2 in pregnancy groups were significantly higher than those in non- pregnancy group ($P<0.01$); and there were significant differences among early pregnancy group, middle pregnancy group and late pregnancy group ($P<0.05$). The proportions of $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^+$ $CD127^-$ Treg in pregnancy groups were significantly higher than those in non- pregnancy group ($P<0.05$), but decreased significantly after parturition, and there was no significant difference between non- pregnancy group and postpartum women group ($P>0.05$); the proportions in middle and late pregnancy groups were significantly higher than those in early pregnancy group ($P<0.05$), but decreased slightly in late pregnancy group, there was no significant difference between late pregnancy group and middle pregnancy group ($P>0.05$). There was correlation between Tregs number with estrogen level during pregnancy. The proportion of $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^+$ $CD127^-$ Treg were positively correlated with estrogen level. **Conclusions:** High proportion of $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^+$ $CD127^-$ Treg is closely related to the high level of E_2 during pregnancy. It suggested that high level of estrogen may induce an increase of $CD4^+$ $CD25^+$ Treg in peripheral blood, and then influence the immune function of pregnant women. The results of this experiment might play an important role of estrogen in immune-modulation during pregnancy.

1. Introduction

$CD4^+$ $CD25^+$ regulatory T cells (Treg) is a group of cells with immunomodulatory or immunosuppressive function, which become a research focus since put forward by Sakaguchi *et al* for the first time in 1995[1], but the regulation factors of this cell is still not clear. Aluvihare[2] firstly reported that $CD4^+$ $CD25^+$ Treg may involved in the regulation of maternal immunotolerance to the fetus. Since then a large number of studies have confirmed that regulatory T cells increased to varying degrees in the maternal peripheral

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blood, the decidua and umbilical cord blood during pregnancy, plays an important role in the maternal-fetal immune tolerance[3-6]. So, there are change factors affecting the number and function of Treg during pregnancy. Large number of experiments *in vitro* and *in vivo* confirmed that estrogen can regulate $CD4^+$ $CD25^+$ Treg, and estrogen significantly increased during pregnancy. Researchers speculate that this factor may be estrogen.

The expression of human $CD4^+$ $CD25^+$ Treg surface markers CD127 was negatively correlated with the suppression function of such T cells, and can replace forkhead/winged helix transcription factor (Foxp3) as the identification of $CD4^+$ $CD25^+$ Treg-specific marker[7]. Therefore, this study detected and analyzed E_2 levels and $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^+$ $CD127^-$ Treg expressions in peripheral blood during pregnancy. This study aims to explore the impact of the changes of the human estrogen on the Treg cells, further elucidate the molecular immune mechanism of the effects of estrogen on immune function, and provide the basis for the exploration of the new method as immunomodulatory therapy.

2. Materials and methods

2.1. Objects

A total of 30 healthy non-pregnant women at early follicular phase were selected as the control group. They aged 22-35 years old, and the average age was 26.5 years. Peripheral venous blood samples were extracted on the day of M3. Ninety women of different pregnancy were selected as study group, in which 30 patients were divided into early pregnancy group, with gestational age 10-12 weeks, aged 20-35 years, average age 27.1 years; 30 patients into middle pregnancy group, with gestational age 26-28 weeks, aged 20-34 years, average age 26.8 years; 30 patients into late pregnancy group, with gestational age 37-40 weeks, aged 21-34 years, average age 26 years. Postpartum group also included 30 patients, aged 22-36 years, with average age 27.2 years. Venous peripheral blood was collected 1 month after delivery.

Cases as following were exclude: (1) who suffer from autoimmune diseases or other immune-related diseases; (2) had a serious infections history within two-years (such as HIV, hepatitis B virus infection); (3) took any immune-modulating agents 6 months before pregnancy; (4) took estrogen or any other sex hormones two years before pregnancy; (5) took corticosteroids during the first two months of pregnancy.

2.2. Instruments and reagents

Fluorescent monoclonal antibody CD4-FITC, CD25-PE, CD127-PE-Cy5, IgG1-FITC, IgG1-PE, IgG1-PE-Cy5 were from EBioscience company. Optilyse erythrocyte lysis buffer, pH 7.4 PBS buffer and Beckman Coulter XL flow cytometer were from Beckman Coulte company, USA. E_2 assay kit and electrochemiluminescence immunoassay analyzer were from Roche company.

2.3. Methods

2.3.1. Specimen Collection

Two mL venous blood was collected at 8 am-9 am. It was placed in EDTA anticoagulant tube and was well mixed for flow cytometry in 4 h. Three mL peripheral venous blood was extracted at the same time. The serum was separated within 4 h and prepared for the estrogen level detection.

2.3.2. Quantitative analysis of Treg

Ten μL of Mab CD4-FITC, CD25-PE, CD127-PE-Cy5 were placed respectively in a clean test tube as the experimental tube; And another 10 μL of IgG1-FITC, IgG1-PE, IgG1-PE-Cy5 were placed in another clean test tube as negative control tube. They were added with 100 μL anticoagulant blood, and then mixed well. They were incubated for 20 min at 4 ℃, added with 400 μL erythrocyte splitting liquor, shocked for 5 s by micro-mixer. Then they were incubated for 15 min at 4 ℃, when red blood cells were fully decomposed, they were centrifuged at 1 500 r/min for 5 min, then supernatant were abandoned. They were washed in 1 mL PBS buffer, added with 400 mL buffer, to resuspendcells in flow cytometry. Lymphocyte populations were selected according to the forward angle and side angle scattering signal, 5×10^4 cells was detected for each sample. $CD4^+$ $CD25^+$ T cells and $CD4^+$ $CD25^+$ $CD127^-$ T cells were calculated by the percentage of $CD4^+$ T cells with flow cytometry Flowjo7.6 software.

2.3.3. Detection of estrogen level

Estrogen was detected by electrochemiluminescence immunoassay. The peripheral blood in the ordinary tube were centrifuged at 3 000 r/min for 10 min. The serum was collected, freezed at -20 ℃, kept the indoor temperature balancing before detection, and detected strictly according to the instruction of kits.

2.4. Statistical analysis

All of the data were analyzed by SSPS17.0 statistics software, and the data are expressed as mean±SD values. Mean comparison between groups was conducted with variance analysis. Correlation was analyzed by Pearson correlation analysis. $P<0.05$ was regarded as statistical significance.

3. Results

The estrogen level in peripheral blood was increased gradually from early pregnancy to late pregnancy, and then decreased significantly after birth. The level 1 month after birth was decreased to the level of non-pregnancy women ($P>0.05$); the levels in pregnancy groups were significantly higher than those in non-pregnancy group ($P<0.01$). There was significant difference in E_2 among early pregnancy group ,middle pregnancy group and late pregnancy group ($P<0.05$). The proportions of $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^+$ $CD127^-$ Treg in pregnancy groups were significantly higher than those in non-pregnancy group ($P<0.05$), but decreased significantly after birth, and there was no significant difference between non-pregnancy group and postpartum women group ($P>0.05$); the proportions in middle and late pregnancy groups were significantly higher than those in early pregnancy group ($P<0.05$), but decreased slightly in late pregnancy group, there was no significant difference between late pregnancy group and middle pregnancy group, maintaining a high standard ($P>0.05$).(Table 1, Figure 1 and Figure 2).

Table 1

$CD4^+$ $CD25^+$ Treg, $CD4^+$ $CD25^+$ $CD127^-$ Treg and E_2 of peripheral blood.

Groups	$CD4^+CD25^+/CD4^+$ (%)	$CD4^+CD25^+CD127^-/CD4^+$(%)	E_2 (pg/mL)
Non-pregnant group	9.47±1.37	3.18±0.40	36.26±5.52
Early pregnancy group	26.13±4.94	8.45±1.92	2 350.44±319.96
Middle pregnancy group	38.02±4.70	12.90±2.14	20 627.80±3 335.50
Late pregnancy group	35.73±3.94	12.11±1.40	45 840.57±6 313.22
Normal puerpera group	9.00±1.29	3.03±0.28	37.96±7.32

There was correlation between Treg level and estrogen level during pregnancy ($r=0.752$, $P<0.01$). The proportions of $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^+$ $CD127^-$ Treg were positively

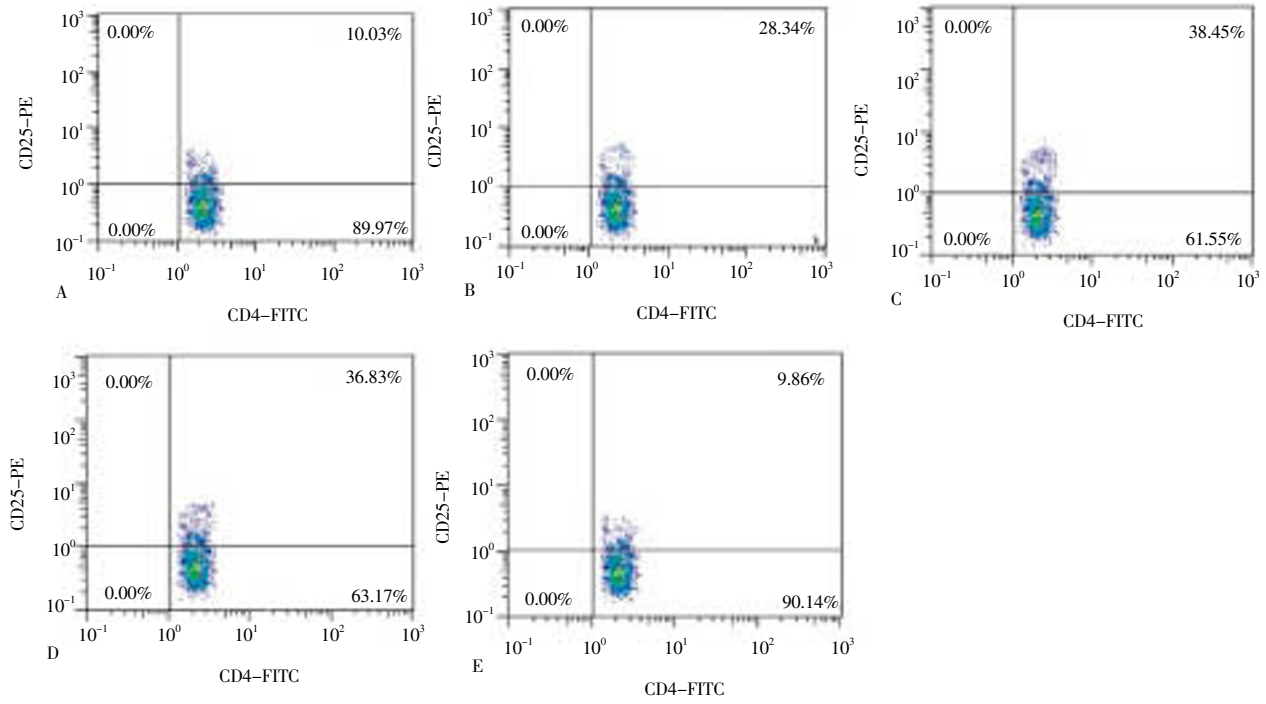

Figure 1. Proportion of $CD4^+$ $CD25^+$ Treg accounted for $CD4^+$ of peripheral blood by FCM in control group (A), early pregnancy group (B), middle pregnancy group (C), late pregnancy group (D) and normal puerpera group (E).

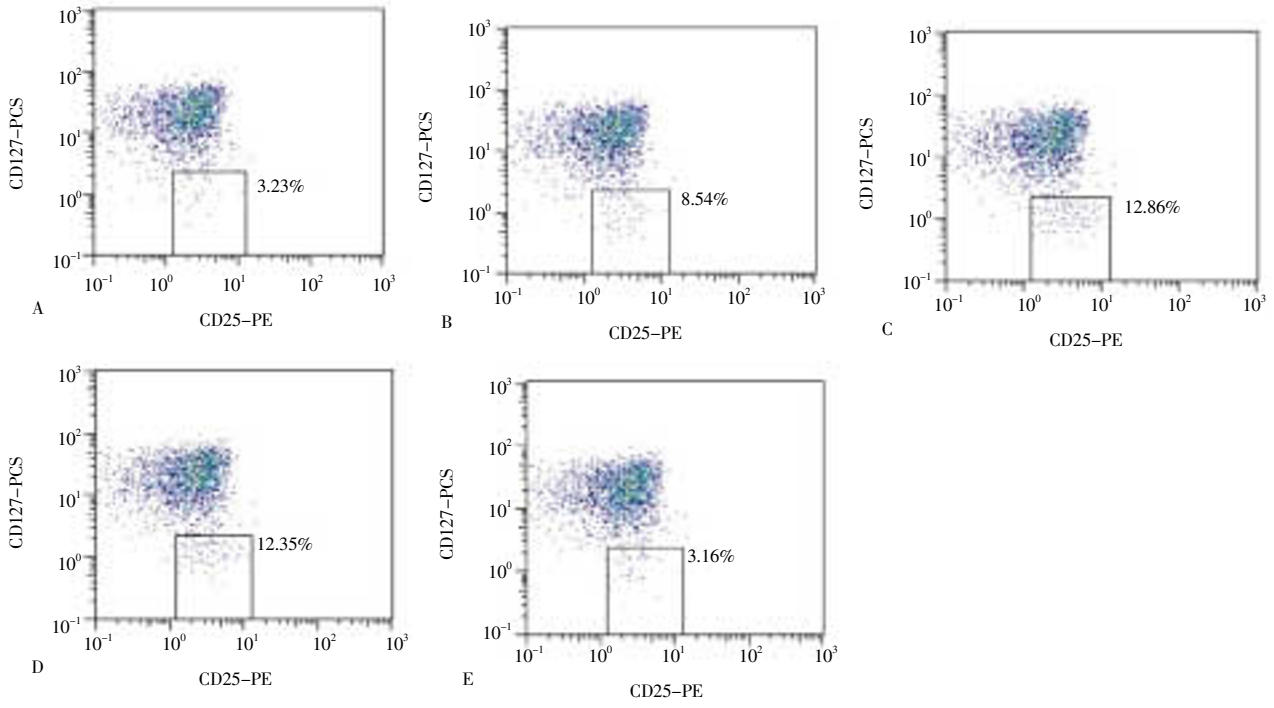

Figure 2. Proportion of $CD4^+$ $CD25^+$ $CD127^-$ Treg accounted for $CD4^+$ of peripheral blood by FCM in control group A, early pregnancy group B, middle pregnancy group C, late pregnancy group D and puerpera group E.

correlated with estrogen level (r=0.746, P<0.01).

4. Discussion

Autoimmune diseases are caused by inappropriate immune response of the body against substances and tissues normally present in the body (autoimmunity). In recent years, the incidence of autoimmune diseases in China is gradually increased, but its pathogenesis is still unknown. There is no effective treatment except for symptoms-relieving in short-term by steroids and immunosuppressive agents. $CD4^+$ $CD25^+$ Treg is a group of cells with functions of immune incompetence and immunosuppressive. Several studies confirmed that the $CD4^+$ $CD25^+$ regulatory T cells plays an important role in the pathogenesis of autoimmune diseases[8-10], decrease or loss of function of such T cells can lead to the occurrence of autoimmune diseases. $CD4^+$ $CD25^+$ regulatory T cells play a key role in the immune tolerance, and scholars at home and abroad believe that it may become the target of the treatment for various autoimmune diseases. It prompts many scholars to study the differentiation processes and factors of Treg cell.

In recent years, the study of the effect of estrogen on the $CD4^+$ $CD2^+$ Treg has become the research focus at home and abroad. Experimental animal studies showed[11] that the $CD4^+$ $CD25^+$ regulatory T cell and Foxp3mRNA and Foxp3 protein expression were significantly increased in pregnant mice and mice treated with exogenous E_2 *in vivo*. Foreign scholars simulated the physique internal environment, then studied the effect of gradient concentration estrogen on normal mouse spleen lymphocyte proliferation. The result showed that physiological doses estrogen can promote the cells proliferation. But with the increase of the estrogen concentration, it began to suppress cells proliferation, and suppression became the stronger as increasing pregnancy concentrations. It confirmed estrogen concentrations under pregnancy can up-regulate the expression of $CD4^+$ $CD25^+$ Treg and the Foxp3mRNA, thereby undermine the dynamic balance between Th1 and Th2 to enhance the immune suppression.

However, these studies were limited to animal experiments and *in vitro* experiments. In this study, we took human as the research object, further confirmed that the estrogen also has a regulatory effect on Treg cells in the human body, and Treg level changed with change in estrogen levels during pregnancy. Proportion of $CD4^+$ $CD25^+$ Treg accounted for $CD4^+$ T cell of peripheral blood of pregnant women was significantly higher than that of non-pregnant control group. With the estrogen levels declining postpartum, the proportion of $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^+$ $CD127^-$ Treg accounted for $CD4^+$ T cell also quickly decreased to non-pregnant levels. It decreased slightly in late pregnancy group, there was no significant difference between late pregnancy group and middle pregnancy group (P>0.05). The high Treg level of

late pregnancy may be because of the maintenance function of high levels estrogen. Treg cells can actively suppress the activation and proliferation of autoreactive T cells, thereby suppress the overreaction of T cell to autoantigens or alloantigens. Autoimmune diseases relieved symptoms during pregnancy and maintained maternal-fetal tolerance. It maybe because that high estrogen levels can increase the level of Treg cells, which inhibit the body's own immune response. Based on this, we resumed that estrogen can adjust the level of T cells to regulate the body's immune response, thus play an important role in the occurrence and development of autoimmune diseases.

$CD4^+$ $CD25^+$ regulatory T cells play a key role in the pathogenesis of autoimmune diseases, various autoimmune diseases all with abnormal number or function of $CD4^+$ $CD25^+$ regulatory T cell. Intervention on the number and function of Treg cells may be the target in treatment of autoimmune diseases and inflammatory diseases. This study confirms estrogen can up-regulate $CD4^+$ $CD25^+$ regulatory T cells, which indicates that Treg level can be adjusted by changing the levels of estrogen in the body.This results might imply an important role of estrogen in immune-modulation during pregnancy. We believe that the effect of estrogen on immune function and their molecular immune mechanism can be better explained in further research.

Conflict of interest statement

We declare that we have no conflict of interest.

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