Effects of regulatory T cells, natural killer cells, and natural killer T cells on immunosuppression therapy in patients with recurrent embryo implantation failure

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Summary
Objective: This retrospective study aims to investigate the effects of immunosuppressive therapy on the expression of regulatory T (Treg) cells, natural killer (NK) cells, and natural killer T (NKT) cells in patients with recurrent embryo implantation failure (RIF). Materials and Methods: In vitro fertilization and embryo transplantation (IVF-ET) at Jinhua Municipal Central Hospital from July 2013 to November 2015 of 30 RIF patients were enrolled into RIF group, and peripheral blood Treg, NK, and NKT were detected before entering the embryo transfer cycle. Meanwhile, 20 normal non-pregnant women were enrolled into control group. Twenty-four patients in RIF group were administered prednisone; Treg, NK, and NKT cell contents were detected twice before and after the embryo transfer cycle, respectively. The remaining six patients with high NK cell contents were treated with gamma globulin via intramuscular injection, and Treg, NK, and NKT cell contents were detected twice before the embryo transfer cycle and after treatment. Results: Treg cell content was significantly lower in RIF group than in control group, while NK cell proportion was significantly higher in RIF group than in control group before treatment. There was no statistical difference in NKT cells. After treatment, expression rate of Treg in RIF group was 7.1 ± 1.8%, which compared with that before treatment (2.8±1.6%) was significantly higher (p < 0.01). In addition, NK cell proportion was significantly lower than pretherapy (20.9 ± 3.6% vs. 38.6 ± 8.1%, p < 0.05) and NKT cell percentage did not present the obvious difference before and after treatment. Conclusion: The occurrence of RIF may be related to the decrease in Treg expression and increase of NK cells. Immunotherapy can upregulate the expression of Treg and decrease NK cell proportion, thereby regulating maternal fetal immune tolerance and reducing the rejection effect of NK cells on fetal foreign bodies, which are conducive to embryo implantation.

Key words: Recurrent embryo implantation failure.; Immunosuppressant treatment; Treg; NK; NKT.

Introduction
Recurrent implantation failure (RIF) in assisted reproductive technology has been a very challenging problem for reproductive scientists and embryologists. In recent years, studies have suggested that RIF and recurrent spontaneous abortion (RSA) may be caused by the same kind of immune dysfunction, and that immune factors play an important role in its pathogenesis [1]. Since the 1980s, many studies on the effects of lymphocytes on immune tolerance in immune-induced women in pregnancy have been carried out, while immunotherapy has been successfully applied to cure RSA patients. However, few studies on RIF have been reported. Studies have confirmed that CD4+CD25+ regulatory T cells (CD4+CD25+ Treg) play an important role in maternal immune tolerance [2], and natural killer (NK) cells play a key role in graft rejection. However, few studies on natural killer T (NKT) cells have been carried out. In this study, Treg, NK, and NKT percentage in peripheral blood of patients before and after immunotherapy treatment were examined using flow cytometry, in order to investigate the application of immunotherapy in RIF, and its effect on pregnancy.

Materials and Methods
This study was conducted in accordance with the declaration of Helsinki in the reproductive center of Jinhua People’s Hospital in Zhejiang Province, China, and received approval from the Ethics Committee of the hospital. Written informed consent was also obtained from all participants.

A total of 30 patients, who received in vitro fertilization and embryo transplantation (IVF-ET) due to fallopian tube obstruction and failed three or more times from July 2013 to November 2015, were enrolled into this study. The already-known factors that caused RIF were ruled out including: patients with abnormalities such as hydrosalpinx, abnormal endocrine (polycystic ovarian syndrome caused by abnormal secretion of sex hormones, as well as hyperthyroidism and hypothyroidism caused by abnormal secretion of thyroid hormones), poor ovarian response, infection histories of TORCH (including Toxoplasma gondii, rubella virus, cytomegalovirus, Herpes simplex virus and others), blood group incompatibility, and immune antibodies such as phospholipid antibodies, endometrial antibody, and antisperm antibody...
were excluded. Endometrium had to show normal morphology under hysteroscopy. Patients were transplanted with high-quality embryos (embryo grading criteria: grade 1: number of cells ≥ 6, debris < 5%, uniform cell sizes, with no obvious cytoplasmic granulation. Grade 2: 6-8 cells, debris < 20%, cells were asymmetric, with mild cytoplasmic granulation. Grade 3: cell number < 6, debris < 50%, with moderate cytoplasmic granulation. Grade 4: cells ceased to develop, debris > 50%, with severe cytoplasmic granulation, and among these, grade 1 or 2 was considered high-quality). RIF patients received immunotherapy one month before the embryo transfer cycle, and the number of treatments was 3-6 times, with an average of 4.15 ± 2.16 times.

A total of 20 normal non-pregnant women who received medical service in this hospital in the same period were enrolled. Patients had histories of pregnancy, but without spontaneous abortion, dead fetus or stillbirths. At the same time, the aforementioned examinations were carried out to exclude chromosomal, anatomic, endocrine abnormalities, infections, and autoimmune diseases.

Phycoerythrin (PE)-labeled mouse anti-human CD4 antibody (CD4-PE, IgG2a), fluorescein iso-thiocyanate (FITC)-labeled mouse anti-human CD25 antibody (CD25-FITC, IgG1) and PE-IgG1, FITC-IgGl of isotype control mice and FACS Canto II flow cytometry were utilized.

Twenty-four of 30 patients in the RIF group were treated with prednisone (5 mg, q.d.) after entering the embryo transfer cycle for six weeks until the 14th day after embryo transfer. The remaining six patients with high NK content were treated with gamma globulin through intramuscular injection at a dose of 0.4 g/kg (body weight) once a day. In the first week of gamma globulin administration, the daily treatment lasted for three consecutive days, and was stopped on the remaining four days. In the second week of gamma globulin administration, the daily treatment lasted for two consecutive days, and was stopped in the remaining five days. In the third week of gamma globulin administration, the treatment lasted for one day, and was stopped in the remaining six days. Peripheral blood was collected to detect the contents of Treg, NK, and NKT cell before and after the transfer cycle, respectively.

Peripheral venous blood was collected (3 ml per head) and anticoagulated with heparin. A double 100-μl heparin-anticoagulated whole blood were placed into two tubes, 5 μl of CD4-PE and CD25-FITC were added into one tube, and 5 μl of PE-IgG2a and FITC-IgG1 isotype controls were added into another tube. These two tubes of samples were mixed well, and incubated in the dark at room temperature for 30 minutes. Then, 500 ml of hemolysin was added, mixed well, incubated in the dark at room temperature for 10 minutes, centrifuged at 1,500 r/min for five minutes, and then the supernatant was discarded. The samples were washed with PBS once, centrifuged at 1,500 r/min for five minutes, and the supernatant was discarded again. Cells were resuspended in 500 μl PBS and immediately tested on the machine.

Data were statistically analyzed by statistical software SPSS 12.0. Data were expressed as mean ± standard deviation (x ± SD). Comparison was performed using t-test, significance level was α = 0.05, and p < 0.05 was considered statistically significant.

Results

Among the RIF patients, 12 were primary infertility patients, while 18 were secondary infertility patients. In the RIF group, patient age ranged from 22 to 35 years, and average age was 29.15 ± 3.66 years. In the control group, the age range of the subjects was from 20 to 35 years, with a mean age of 29.65 ± 3.88 years. The difference in age between the two groups was not statistically significant (r = 1.97).

Before the immunotherapy treatment, the difference in the proportion of peripheral blood CD4+ T cells in lymphocytes between the RIF and control groups was not sta-
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...ntistically significant (p > 0.05). Furthermore, the proportion of CD4⁺CD25⁺ Treg cells in CD4⁺ T cells showed significantly reduction in the RIF group than in the control group (2.8 ± 1.6% vs. 6.6 ± 2.1%, p < 0.01), while the proportion of NK cells in peripheral blood was significantly higher in the RIF group than in the control group (38.6 ± 8.1% vs. 18.9 ± 3.7%, p < 0.01, Table 1). In the RIF group, the difference in the proportion of CD3⁻TCRVα24⁻TCRVβ11⁻ NKT cells in lymphocytes before and after treatment was not statistically significant (p > 0.05, Table 1). Results of flow cytometry are shown in Figures 1, 2, and 3. In Figure 1 a representative dot plots show the population change of

Figure 2. — Gating strategy for flow cytometric identification of NKT (CD3⁺TCRVα24⁺TCRVβ11⁺) cells in the peripheral blood. (A) A representative CD3-PerCP Cy5.5 gating is set for T lymphocytes from peripheral blood before gamma globulin treatment. (B) A representative dot plot showing expression of NKT (CD3⁺TCRVα24⁺+TCRVβ11+) cells (Q2) identified by setting TCRVα24-FITC and TCRVβ11-PE gating before gamma globulin treatment. (C) A representative CD3-PerCP Cy5.5 gating is set for T lymphocytes from peripheral blood after gamma globulin treatment. (D) A representative dot plot showing expression of NKT (CD3⁺TCRVα24⁺TCRVβ11⁺) cells (Q2) identified by setting TCRVα24-FITC and TCRVβ11-PE gating after gamma globulin treatment.

Figure 3. — Gating strategy for flow cytometric identification of Treg (CD4⁺CD25⁺CD127low) cells in the peripheral blood. (A) A representative CD4-FITC gating is set for Th lymphocytes from peripheral blood before gamma globulin treatment. (B) A representative dot plot showing expression of Treg (CD4⁺CD25⁺CD127low) cells (green) identified by setting CD25-APC and CD127-PE gating before gamma globulin treatment. (C) A representative CD4-FITC gating is set for Th lymphocytes from peripheral blood after gamma globulin treatment. (D) A representative dot plot showing expression of Treg (CD4⁺CD25⁺CD127low) cells (green) identified by setting CD25-APC and CD127-PE gating after gamma globulin treatment.
CD3/CD16+56 NK cells in the peripheral blood of the women with RIF before and after treatment. Additionally, Figure 2 shows the percentage of CD3+TCRVα24/β11+NKT cells among the peripheral blood of the women with RIF and Figure 3 shows the presence of CD4+CD25+CD127low Treg cells in the patients with RIF before and after gamma globulin therapy.

The expression rate of CD4+CD25+ Treg cells in the RIF group showed an significantly increase higher after treatment compared with that of before treatment (7.1 ± 1.8% vs. 2.8 ± 1.6%, p < 0.01). Nevertheless, the percentage of NK cells in the patients with RIF reduced significantly (20.9 ± 3.6% vs. 38.6 ± 8.1%, p < 0.05) and NKT cell did not show a statistical difference (0.28 ± 0.13 vs. 0.31 ± 0.26, p > 0.05) after treatment. The results are shown in Table 2.

### Table 2. — Comparison of Treg, NK, and NKT in peripheral blood in the RIF group before and after treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Treg</th>
<th>NK</th>
<th>NKT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td>30</td>
<td>4.0±1.6</td>
<td>38.6±8.1</td>
<td>0.31±0.26</td>
</tr>
<tr>
<td>After treatment</td>
<td>30</td>
<td>7.1±1.8</td>
<td>20.9±3.6</td>
<td>0.28±0.13</td>
</tr>
</tbody>
</table>

Note: Treg in the RIF group is significantly higher after treatment than before treatment, and the difference was statistically significant (p < 0.05). NK in the RIF group is significantly higher after treatment than before treatment, and the difference is statistically significant (p < 0.05).

The present authors also found that natural killer (NK) cell populations were higher than healthy subjects and significantly decreased after immunosuppressive therapy. It is clear that women with reproductive failure have abnormal NK cell parameters, reflecting high immunological activity. Suppressing NK cell overactivity may be conducive to assisted reproduction failure. Evaluating NK population as CD3+CD16+CD56-, the authors confirmed data obtained in another study [5].

CD4+CD25+ Treg cells are T-lymphocyte subsets with immunosuppressive regulatory function. CD4+CD25+ Treg cells accounted for 5-10% of blood CD4+ T lymphocytes, which inhibit the activation, proliferation, and effector function of reactive CD4+ T lymphocytes or cytotoxic CD8+ T lymphocytes [6, 7]. These cells play an important role not only in preventing autoimmunity, but also in regulating tumor immunity and inducing transplantation tolerance [8-10]. Zencussen [11] and Shima et al. [12] reported that maternal CD4+CD25+ Treg cells can suppress the semi-allo-antigen-induced maternal immune rejection to the embryo, and play an important role in maintaining pregnancy immune tolerance during embryo implantation and early pregnancy. Heikkinen et al. [13] confirmed that during pregnancy, the amount of CD4+CD25+ Treg cells in peripheral blood increased, and since the beginning of the first trimester, CD4+CD25+ Treg cells migrate from the peripheral blood to the mother-fetus interface. Jasper et al. [14] found in detection by reverse transcription polymerase chain reaction (RT-PCR) that the expression of CD4+CD25+ Treg in secretory phase endometrium was significantly lower in RIF patients than in normal non-pregnant women. In this study, flow cytometry results revealed that CD4+CD25+ Treg content in peripheral blood was significantly lower in the 30 patients with RIF before entering the embryo transfer cycle than in normal controls, and the difference was statistically significant (p < 0.05).

NK cells belong to the lymphocyte lineage, also known as large granular lymphocytes. NK cells have cytotoxic effect, and can spontaneously kill target cells without presensitization by antigens. NK cells were mainly distributed in peripheral blood, accounting for 10-15% in peripheral blood lymphocytes. They can transfer through the blood circulation to various tissues and take effects. NK cells are the most important lymphocyte population in the uterus. Since decidua NK (uNK) cells can direct contact with fetal trophocytes, if the cytotoxicity of uNK cells becomes excessive, uNK cells would likely become a fatal factor for the embryo. Since the first report by Aoki, abnormalities in the number and function of NK cells have been considered to be important factors leading to RSA [15, 16]. In the present study, flow cytometry results revealed that NK cell content in peripheral blood before treatment was higher in the RIF group than in the control group, and the difference was statistically significant. Experimental results revealed that the increased proportion of NK cells in peripheral blood...
was the reason for the failure of IVF-ET.

In 1987, another special cell group was found in which such cells simultaneously expressed NKI antigens, and NK1 T cell receptor (TCR) Vαβ, was called NKT cells. Studies revealed that NKT cells have a very close relationship with the maternal-fetal interface, and it is speculated that NKT cells can induce a Th2-type micro-environment via interleukin-4 (IL-4), thus playing an important role during the peri-implantation period [17, 18]. Wang et al. [2] detected a time-related expression of NKT cells in uterine decidua and found that NKT cells exhibited a high expression in the early stage of pregnancy and a relatively lower expression in the late stage of pregnancy, in which they indicated that the decrease in the number of NKT cells in the later stage of pregnancy may be important for the maintenance of pregnancy [19]. In the present study, flow cytometry results revealed that NKT cell content in peripheral blood before treatment was slightly higher in the RIF group than in the control group, but the difference was not statistically significant.

The Treg, NK and NKT content in peripheral blood in the 30 patients with three or more IVF-ET failures before entering the embryo transfer cycle were analyzed. The present authors failed to detect Treg, NK, and NKT contents in the decidua. Treg, NK, and NKT content in peripheral blood may not fully reflect the contents of these three in the decidua; hence, this method has some limitations. Experimental studies have revealed that Treg content was lower in the experimental group than in the control group, and NK content was higher in the experimental group than in the control group; however NK content had no significant difference compared with the control group. After prednisone treatment (gamma globulin treatment in patients with high NK), Treg cell level significantly increased after treatment than before it. Experiments revealed that the downregulated expression of Treg cells and increased expression of NK cells are possible factors that lead to IVF-ET failure. Furthermore, immunosuppressive therapy may have corrected the expression of Treg and NK cells to a certain extent. Pregnancy outcomes require further controlled studies.

References

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