

ORIGINAL ARTICLES

Animal study on CD4⁺ CD25⁺ regulatory T cells for treating female mouse with recurrent spontaneous abortion

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Received: August 3, 2015

Accepted: October 15, 2015

Online Published: December 10, 2015

DOI: 10.14725/dcc.v2n4p1

URL: <http://dx.doi.org/10.14725/dcc.v2n4p1>

Abstract

Objective: To explore immunotherapy effectiveness of the CD4⁺ CD25⁺ regulatory T cells for treating female mouse with recurrent spontaneous abortion (RSA) by animal experiments.

Methods: Mononuclear lymphocytes were isolated from the blood (instead of cord blood) of new-born baby of KunMing Bai mouse or BALB/c male mouse with normal birth ability (as unrelated third party blood source) by density gradient centrifugation method. The CD4⁺ CD25⁺ regulatory T cells were selected by magnetic-activated cell sorting from mononuclear cells of cord blood cells. CBA/J female mouse copulated with DBA/2J male mouse was utilized as RSA animal model. Pregnant RSA mice were injected different types of lymphocytes through tail vein. Independent sample *t*-test was used to analyze the data from each group.

Results: The proportion of CD4⁺ CD25⁺ T cells in CD4⁺ T cells was (17.49 ± 0.60)% in CD4⁺ CD25⁺ regulatory T cells injection group, which was statistical significant higher than that of mononuclear lymphocyte injection group (14.68 ± 0.83)%, sterile PBS group (9.54 ± 0.85)% or no injection group (9.28 ± 0.68)% (*p* < .05, *t*-value was 4.754, 13.242 and 15.621, respectively). The Foxp3 relative protein expression level of CD4⁺ CD25⁺ regulatory T cells injected group was 5.85 ± 0.45, which was also significant higher than that of mononuclear lymphocyte injection (2.86 ± 0.54), sterile PBS group (1.08 ± 0.16) or no injection group (1.00 ± 0.00) (*p* < .05, *t*-value was 7.276, 17.227 and 18.635, respectively). Finally, two times of CD4⁺ CD25⁺ T cell injected group at the 4th and 8th day had well effect for RSA mouse, and embryo sorption rate was (4.92 ± 0.08)%, which significant lower than that of two times of mononuclear lymphocyte injected group (13.07 ± 0.06)%, sterile PBS group (23.11 ± 0.12)%, or no injection group (25.47 ± 0.11)% (*p* < .05, *t*-value was -2.603, -4.012 and -4.700, respectively).

Conclusions: Pregnant mouse with RSA injected CD4⁺ CD25⁺ T cells several times for immunotherapy can get better effectiveness than that of pregnant mouse injected traditional mononuclear cells.

Key Words: Regulatory T cells, Recurrent spontaneous abortion, Immune therapy, Immune tolerance

Clinically, a paternal or an unrelated third party blood cells isolated from peripheral blood are used for active immunotherapy to the patients with recurrent spontaneous abortion (RSA) caused by isoimmune disorder. Although active immunotherapy can improve the pregnancy rate of

patients with RSA due to negative antibody,^[1] as the complicated complications of RSA, there exist some limitations about traditional treatment methods. There is some evidence to suggest that the number and function of T cells in vivo in the RSA patient is significantly reduced, making

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Th1 immune response too strong and the Th2 type immune response insufficient, which may lead to the occurrence of miscarriage.^[2,3] Therefore, the artificial supplement of the regulation of T cells in the body may become a new strategy for the treatment of RSA. CD4⁺ CD25⁺ regulatory T cells are a class of CD4⁺ T ancillary cells with immunoregulatory regulation, and their surface express IL-2 receptor (CD25).^[4] At present, the proportion of regulatory T cells obtained from adult peripheral blood is low (1% to 2%), which hinders regulation T cells for immunotherapy.^[5] In contrast, a large proportion of CD4⁺ CD25⁺ regulatory T cell subsets can be obtained from umbilical cord blood, and the resulting cells are in a relatively primitive state and are not easily stimulated into effector T cells in vitro.^[6] It provides a great convenience for clinical application studies. In this study, we investigated CD4⁺ CD25⁺ regulatory T cells for immunotherapy in RSA patients by animal experiments, which provided new therapeutic ideas for the treatment of RSA.

1 Materials and methods

1.1 Materials

Ficoll-Hypaque was produced by Amersham Bioscience; Immunomagnetic beads separation kit was purchased from MiltenyiBiotec; RPMI1640 medium was purchased from Invitrogen-Gibco; Phosphate buffer (PBS) was purchased from Hyclone; 8% paraformaldehyde was purchased from Electron Microscopy Sciences; Triton X-100 and trypan blue were produced by Sigma; Tween 20 and bovine serum albumin (BSA) were produced by Amresco; Various antibodies were produced by the Abcam/Jackson ImmunoResearch Company; Kunming white female mice (6 weeks old, SPF grade, 22.7 ± 2.2 g) and male mice (8 weeks old, SPF grade, 28.9 ± 2.6 g) were purchased from Inner Mongolia University Experimental Animal Center; Female CBA/J mice (6 weeks old, SPF grade, 19.3 ± 1.8 g), male DBA/2J mice (8 weeks old, SPF grade, 24.3 ± 2.4 g) and male BALB/c mice (8 weeks old, SPF grade, 25.4 ± 1.5 g) were purchased from Model Animal Research Institute of Nanjing University.

1.2 Isolation and screening of CD4⁺ CD25⁺ positive regulatory T cells from cord blood

10 ml blood of Kunming white mice was collected and placed in 15 ml anticoagulated sterile centrifuge tube. Ficoll-Hypaque density gradient method was used to separate mononuclear cells.^[6] The umbilical cord blood was diluted with sterile PBS (1:1) and was placed above 10 ml of Ficoll-Hypaque solution; Centrifuged for 25 minutes at 400×g, and then recycled the white cell layer. CD4⁺ T cells were enriched and subjected to column treatment

with immunomagnetic beads separation kit (mixed antibody containing CD8, CD11b, CD16, CD19, CD36 and CD56). The total positive cells were separated by anti-CD25⁺ magnetic beads, and 10 μl anti-mouse CD25-PE/10⁶ mononuclear cells were added. Ice incubation for 30 min. Centrifuged for 10 min at 300×g, then resuspended cells in 250 μl MACS buffer resuspended cells; added 10 μl anti-PE MACS beads, ice incubation for 30 min, 300×g, 10 min centrifugation; resuspended cells in 500 μl MACS buffer, and recycled the positive cells after passing column.

1.3 Isolation of mononuclear lymphocytes from peripheral blood of BALB/c male mice

In order to simulate the immunotherapy of human recurrent miscarriage, BALB/c male mice were selected as unrelated third-party blood sources, and mononuclear lymphocytes in the blood were separated by Ficoll-Hypaque density gradient method.

1.4 Establishment of repeated abortion mouse model

CBA/J female mice × DBA/2J male mice mating combination are often considered as repeated spontaneous abortion animal model. The abortion rate ranges from 20% to 40%, which is relatively constant.^[7] Mating and pregnancy were determined: abortion group was CBA/J female × DBA/2J male mice, the control group was CBA/J female × BALB/C male mice. Two groups of mice were placed in the same cage according to male: female = 1: 2 ratio in the quasi-mating day at 17:30 p.m. after weighing. The vaginal suppository was checked once at 8:00 a.m. and 20:00 p.m. the next day, sub-cage feeding after seeing bolt (marked as pregnancy for 0.5 d).

1.5 Cord blood-regulatory T-cells injection of RSA mouse model

RSA pregnant mice were randomly divided into 7 groups. The number of groups 1, 2, 3 and 7 is 15, the number of groups 4, 5, 6 is 10 respectively. Pregnant mice in groups 1, 2 and 3 were separately given tail vein injection of T lymphocyte [(2-2.5) × 10⁶/ml, 50 μl], male BALB/c mice peripheral blood mononuclear lymphocytes [(2-2.5) × 10⁶/ml, 50 μl] and sterile PBS (blank control); Mice in groups 4, 5 and 6 were separately given tail vein injection of T lymphocytes [(2-2.5) × 10⁶/ml, 50 μl], male BALB/c mice peripheral blood mononuclear lymphocytes [(2-2.5) × 10⁶/ml, 50 μl] and sterile PBS; group 7 was not injected.

1.6 Detection of CD4⁺ CD25⁺ T cells

Flow cytometry was used to detect spleen CD4⁺ CD25⁺ T cells. The spleen mononuclear cells obtained from 5 pregnant rats in the above groups 1, 2, 3 and 7 were centrifuged and washed with sterile PBS. The total number of cells were $(5-6) \times 10^7$ each mouse. Cells were incubated with 10 $\mu\text{g/ml}$ FITC-labeled anti-CD4 and 5 $\mu\text{g/ml}$ PE-labeled anti-CD25 monoclonal antibody, and the cells were thoroughly mixed for flow cell sorting.

1.7 Detection of Foxp3 expression in CD4⁺ CD25⁺ T cells

The expression of Foxp3 in CD4⁺ CD25⁺ T cells was detected by Western Blot. The total protein was extracted from CD4⁺ CD25⁺ T cells, and the protein content was determined by SDS-PAGE. Transferred to the membrane and made the immune response. The primary antibody was anti-rabbit-Foxp3 antibody and anti-mouse- β -Actin, and the dilution ratio was 1:500. The secondary antibody was HRP-

donkey anti-rabbit antibody and HRP-donkey anti-mouse antibody. The dilution ratio was 1:10,000.

1.8 Observation of embryonic development

On the 13th day of gestation, 10 CBA/J pregnant mice in each group were sacrificed, the numbers of embryos absorbed and surviving embryos were calculated. The rate of embryo absorption was calculated according to the formula (number of embryos absorbed/number of embryos \times 100%).

1.9 Statistical analysis

SPSS 17.0 software was used for statistical analysis. Independent samples were used to compare the CD4⁺ CD25⁺ T cell ratio, Foxp3 expression rate and different injection methods of embryo absorption rate. Test degree is $\alpha = 0.05$ (both sides).

Table 1: The results of CD4⁺ T and CD4⁺ CD25⁺ T cells in each group (% , $\bar{x} \pm s$)

Groups	The number of mice	CD4 ⁺ T cells	CD4 ⁺ CD25 ⁺ T cells	CD4 ⁺ CD25 ⁺ T cells proportion
1	5	25.17 \pm 0.51	4.40 \pm 0.10	17.49 \pm 0.60
2	5	25.20 \pm 0.10	3.70 \pm 0.20	14.68 \pm 0.83*
3	5	24.47 \pm 0.15	2.33 \pm 0.21	9.54 \pm 0.85**
7	5	25.17 \pm 0.40	2.33 \pm 0.15	9.28 \pm 0.68**

Note. * Compared to group 1, $p < .05$; # Compared to group 2, $p < .05$

2 Results

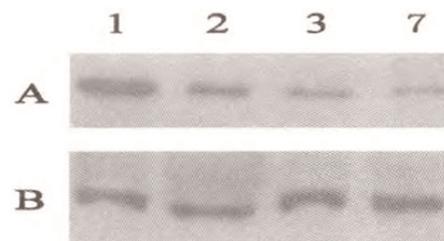
2.1 The proportions of CD4⁺ CD25⁺ T cells

Compared with groups 2, 3 and 7, the differences were statistically significant shown in group 1 ($t = 4.754, p < .05$; $t = 13.242, p < .05$; $t = 15.621, p < .05$). The differences between group 2 and groups 3, 7 were statistically significant ($t = 7.529, p < .05$; $t = 8.737, p < .05$). There was no significant difference between group 3 and group 7 ($t = 0.415, p > .05$) (see Table 1).

2.2 Expression of Foxp3 in CD4⁺ CD25⁺ T cells

The gray scale of Western Blot results was analyzed by Image J software. The gray scale values were compared with those of group 7. The relative expression of protein was obtained. The results showed that the expression of Foxp3 in group 1 (5.85 ± 0.45) was significantly higher than that in group 2 (2.86 ± 0.54), group 3 (1.08 ± 0.16) and group 7 (1.00 ± 0.00), the differences were statistically significant ($t = 7.276, p < .05$; $t = 17.227, p < .05$; $t = 18.635, p < .05$); There were significant differences between group 2 and groups 3, 7 ($t = 5.385, p < .05$; $t = 5.871, p < .05$).

There was no significant difference between group 3 and group 7 ($t = -0.846, p > .05$) (see Figure 1).



A: Expression of Foxp3 B: Expression of β -Actin as an internal control gene

Figure 1: The detection of Foxp3 expression by Western Blot

2.3 Observation of embryonic development

The differences between group 4 and groups 5, 6, 7 were statistically significant ($t = -2.603, p < .05$; $t = -4.012, p < .05$; $t = -4.700, p < .05$). There was no significant difference between group 1 and groups 2, 3. Compared with group 7, the difference was statistically significant in group 1 ($t = -2.177, p < .05$) (see Table 2).

Table 2: The comparison of embryonic absorption in each group

Groups	The number of mice	The total number of embryos absorbed/The number of embryos	Absorption rate of embryos (% , $\bar{x} \pm s$)
1	10	8/70	12.42 \pm 0.15*
2	10	13/75	15.91 \pm 0.10
3	10	16/64	25.80 \pm 0.15
4	10	4/69	4.92 \pm 0.08
5	10	12/72	13.07 \pm 0.06 [#]
6	10	17/72	23.11 \pm 0.12 [#]
7	10	18/69	25.47 \pm 0.11 [#]

Note. * Compared to group 7, $p < .05$; [#] Compared to group 4, $p < .05$

3 Discussions

Isoimmune-type RSA accounts for a large proportion in the incidence of RSA, which is in the same genus for different antigenic components of the immune response occurred, about 50% RSA patients can not find clear causes. Pregnancy is equivalent to allograft,^[8] the fetus carrying paternal HLA antigen can stimulate the maternal immune activity cells resulting in immune response; at the same time, stimulated by the embryo carried by the paternal antigens, the immunosuppressive cells in the body of the mother are also increase, so as to maintain immune tolerance. The immune response and immunosuppression keep a balance circumstance for fetal survival and delivery. CD4⁺ CD25⁺ regulatory T cells have the characteristics of negative regulation of immune function and low reactivity, and play an anti-inflammatory effect and maintain autoimmune tolerance through intercellular contact inhibition and secretion of inhibitory cytokines. Foxp3 is a molecule marker and specific regulator that plays an important role in regulating the development of T cells in the thymus, the distribution of peripheral blood and the maintenance of immunosuppressive function. Studies have confirmed that the newly discovered IL-35, a rare immunosuppressive cytokine secreted by regulatory T cells, regulates the activity of T cells to achieve the purpose of immunosuppression.^[9]

In this study, we compared the effect of CD4⁺ CD25⁺ T cells and traditional immunotherapy with mononuclear lymphocytes for recurrent miscarriage. Flow cytometry results showed that CD4⁺ CD25⁺ T cells in group 1 were significantly higher than those in group 2 (mononuclear lymphocytes group), group 3 (PBS group) and group 7 (non-injected group), indicating that injection of CD4⁺ CD25⁺ T cells could produce more regulation of T cells than injection

with mononuclear lymphocytes.

Traditional injection of mononuclear lymphocytes also resulted in an increase in regulatory T cells compared to the blank control group and the non-injected group, which might explain why the traditional method was effective.^[10] Western Blot test showed that CD4⁺ CD25⁺ T cells injection group expressed more Foxp3 protein. Foxp3 is the specific regulation of T cells, injection of CD4⁺ CD25⁺ T cells seems to promote the body to express more Foxp3 protein, which is more conducive to play an immunosuppressive function.^[11] The results of this study showed that the embryonic absorption rates in CD4⁺ CD25⁺ T cells injection group (group 4) were significantly lower than those in mononuclear lymphocytes group (group 5), group 6 (PBS injection for twice) and non-injected group (group 7) at the 4th and 8th day, indicating that two injections of T-cell after pregnancy were more suitable for RSA than conventional injection of mononuclear lymphocytes.^[12] There was no difference in the single injection of CD4⁺ CD25⁺ cells from the single injection of mononuclear lymphocytes and the injection of PBS on day 4, indicating that single injection of CD4⁺ CD25⁺ regulatory T cells did not gain a therapeutic advantage.

4 Conclusions

Animal experiments suggested that the CD4⁺ CD25⁺ regulatory T cells supplementation might be a new strategy for the treatment of RSA.

Conflicts of Interest Disclosure

The authors have no conflicts of interest related to this article.

References

[1] Kling C, Steinmann J, Flesch B, et al. Transfusion-related risks of intradermal allogeneic lymphocyte immunotherapy: single cases in a large cohort and review of the literature. *Am J Reprod Immunol.* 2006; 56(3): 157-171. PMID: 16911711. <https://doi.org/10.1111/j.1600-0897.2006.00413.x>

[2] Saito S, Sasaki Y, Sakai M. CD4+CD25+regulatory T cells in human pregnancy. *J Reprod Immunol.* 2005; 65: 111-120. PMID: 15811516. <https://doi.org/10.1016/j.jri.2005.01.004>

- [3] Yang H, Qiu L, Chen G, et al. Proportional change of CD4+CD25+regulatory T cells in deciduas and peripheral blood in unexplained recurrent spontaneous abortion patients. *Fertil Steril*. 2007; 89(3): 656-661. PMID: 17543960. <https://doi.org/10.1016/j.fertnstert.2007.03.037>
- [4] Sakaguchi S, Sakaguchi N, Asano M, et al. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995; 155(3): 1151-1164.
- [5] Parmar S, Liu X, Tung SS, et al. Third-party umbilical cord blood-derived regulatory T cells prevent xenogenic graft-versus-host disease. *Cytotherapy*. 2014; 16(1): 90-100. PMID: 24480547. <https://doi.org/10.1016/j.jcyt.2013.07.009>
- [6] Bresatz S, Sadlon T, Millard D, et al. Isolation, propagation and characterization of cord blood derived CD4+CD25+regulatory T cells. *J Immunol Methods*. 2007; 327(1-2): 53-62. PMID: 17804010. <https://doi.org/10.1016/j.jim.2007.06.006>
- [7] Clark DA, McDermott MR, Szewczuk MR. Impairment of host-versus-graft reaction in Pregnant mice. II. Selective suppression of cytotoxic T-cell generation correlates with soluble suppressor activity and with successful allogeneic Pregnancy. *Cell Immunol*. 1980; 52(1): 106-118. [https://doi.org/10.1016/0008-8749\(80\)90404-9](https://doi.org/10.1016/0008-8749(80)90404-9)
- [8] Makrigiannakis A, Petsas G, Toth B, et al. Recent advances in understanding immunology of reproductive failure. *J Reprod Immunol*. 2011; 90(1): 96-104. PMID: 21683452. <https://doi.org/10.1016/j.jri.2011.03.006>
- [9] Castellani ML, Anogeianaki A, Felaco P, et al. IL-35, an anti-inflammatory cytokine which expands CD4+CD25+Treg Cells. *J Biol Regul Homeost Agents*. 2010; 24(2): 131-135. PMID: 20487626.
- [10] Szekeres-Bartho J. Progesterone-mediated immunomodulation in pregnancy: its relevance to leukocyte immunotherapy of recurrent miscarriage. *Immunotherapy*. 2009; 1(5): 873-882. PMID: 20636029. <https://doi.org/10.2217/imt.09.54>
- [11] Wang XJ, Leveson-Gower D, Golab K, et al. Influence of pharmacological immunomodulatory agents on CD4(+) CD25(high) FoxP3(+)T regulatory cells in humans. *Int Immunopharmacol*. 2013; 16(3): 364-370. PMID: 23499512. <https://doi.org/10.1016/j.intimp.2013.02.015>
- [12] Wu L, Luo LH, Zhang YX, et al. Alteration of Th17 and Treg cells in patients with unexplained recurrent spontaneous abortion before and after lymphocyte immunization therapy. *Reprod Biol Endocrinol*. 2014; 12: 74. PMID: 25086467. <https://doi.org/10.1186/1477-7827-12-74>