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Original Article

Combination of $CD4^+CD25^+CD127^-$ regulatory T cells with MLC-BE and BE-Ab2: an efficient evaluation of the therapy of paternal lymphocyte induced immunization in unexplained recurrent spontaneous abortion patients

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Abstract: The aim of this retrospective study was to compare the immune tolerance status of patients suffered from unexplained spontaneous abortion (URSA) before and after treatment with paternal lymphocyte induced immunization (PLII) four times, and its relationship to the pregnancy outcome. 168 URSA patients were included in the present study. Among 168 couples, 138 couples were conceived again, of whom 86 were successfully pregnant till 20 gestational weeks, 31 cases again failed in the first trimester, 21 cases were still under follow-up, another 30 cases still had not conceived. Both the level of one way mixed lymphocyte culture blocking efficiency (MLC-BE) and anti-idio blocking antibody (BE-Ab2) were markedly elevated in succeeded group after PLII. In contrast, although a significant increase could be observed in the failed group after treatment, the elevation of BE-Ab2 was much lower than that in successful group. PLII therapy significantly up-regulated the percentage of peripheral $CD4^+CD25^+CD127^-$ regulatory T cells (Tregs) in successfully pregnant women; however, there was no significant change of Tregs in pregnancy loss cases although receiving PLII therapy. These results suggested a positive correlation between higher frequency of Tregs and rate of successful pregnancies. The sensitivity and specificity of combination of Tregs with MLC-BE and BE-Ab2 were 81.8% and 81.3%, respectively. Therefore, the percentage of Tregs in peripheral blood may hopefully serve as a potential biomarker for monitoring the efficacy of therapy in URSA patients. Combination of Tregs with MLC-BE and BE-Ab2 may expect to better evaluate the efficacy of PLII in URSA patients.

Keywords: URSA, PLII, MLC-BE, BE-Ab2, Tregs, blocking antibody

Introduction

In recent decades immunologists have reached a consensus that pregnancy is a unique immunological state in which a balance of immune tolerance and suppression is needed to protect the fetus from rejecting. Successful pregnancy is actually an extraordinary challenge and immunologic paradox which permits the conceptus figuratively as a semi-allograft accepted by the mother. About 1%-5% of fertile couples are frustrated by suffering repeated pregnancy loss which usually occurs in the first trimester or before the 20th gestational week [1]. Maintaining the pregnancy involves a series of mechanisms including balance of Type 1 (Th1) and Type 2 (Th2) helper T cells; interaction of CD95 and its ligand; expression of the placenta HLA (human leukocyte antigen), etc. Unexplained recurrent spontaneous abortion (URSA) is largely associated with the failure of feto-maternal immunologic tolerance [2]. Except the widely accepted multifactorial etiological reasons including aspects of chromosomal, anatomic, endocrinological, infectious, autoimmunologic abnormalities, environmental, and psychological factors, etc. The underlying cause of about 50% URSA which is so called immunologically mediated URSA still remains unidentified. Improper recognizing of paternally inherited alloantigens of the conceptus by the immunological system of the mother leads to inability to develop a protective response. Accordingly, induction a state of maternal immuno-suppression to the fetus is a main target for maintaining maternal-fetal tolerance. It was revealed from

extensive updated analysis that effective presentation of paternal alloantigens achieved by immunization of the mother with paternal lymphocytes may play a significant role in the prevention of URSA [3].

Although the precise mechanism that underlines the benefits of this immunotherapy still keeps obscure, treatment for URSA by alloimmunization of women with paternal or third-party lymphocyte transfusion to induce suppression of the maternal immune responses to the allogeneic fetus *in vitro* as an effective mode has been recommended since 1985 [4]. Since then on, lymphocyte immunization has been a widely accepted immunological intervention for the treatment of URSA in some countries. In China, this kind of treatment was becoming quite widespread in the past decade, so exploring and establishing the appropriate diagnostic method or reliable indicator for monitoring the efficacy of paternal lymphocyte induced immunization (PLII) therapy were of particular importance.

As it has long been established that a delicate balance of Th1-Th2 cytokines directed toward a Th2-dominant pattern is considered a very important mechanism in favor of pregnancy maintenance, immune cells such as natural killer (NK) cells, and some cytokines such as interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α which reflect the Th1 level, as well as interleukins of IL-4, IL-6, IL-10 and IL-13 which reflect the Th2 profile, are theoretically reasonable for evaluating the efficacy of the immunotherapy. But these cells are known to act mainly locally in the endometrium and decidua, so the measurements of cytokines in peripheral blood were of limited significance. Currently, blocking antibody (BA) is one of the most commonly used parameters and approved as a relatively acceptable monitoring indicator for diagnosis and treatment effectiveness of URSA. Most laboratories in China examine the blocking antibody indirectly by enzyme linked immunosorbent assay (ELISA), complement dependent cytotoxicity (CDC) or flow cytometry (FCM). For detecting BA, ELISA and CDC showed week correlation, the two methods reflect exactly not the same antibody composition and represent diagnostic value respectively [5]. Furthermore, all the above detection of BA was directed against specific HLA antigen, which was poorly reproducible between laboratories. Another disputable view is that anti-HLA antibodies in pregnant women are considered to be the consequences instead of causes of successful pregnancies. Therefore, the question as to whether BA is efficient in the evaluation of immunotherapy was currently kept controversial. In this study, we detect the blocking effect directly by one-way mixed lymphocyte reaction (MLC-BE), and expected to elucidate the efficacy of BA by exploring the relationship between MLC-BE positive conversion rates and pregnant results after immunization.

The $CD4^+CD25^+$ regulatory T cells were described as a unique subpopulation of T cells, known to play an important role in the development and maintenance of tolerance in peripheral tissues. The potential role of $CD4^+CD25^+$ Treg cells in maintaining human pregnancy preventing from fetal rejection has been reported and demonstrated through mice model experiments [6]. In accordance with this, recent publications revealed that the proportion of $CD4^+CD25^+$ Treg cells was down-regulated during human miscarriage, and was elevated after lymphocyte immunotherapy [7]. Foxp3 is a master regulator gene for the development and function of Tregs, its deficiency was proven to consequently impair the suppressive function of Treg cells. It has been proposed that reduction of Treg cells in URSA patients is closely related to the decreased expression of Foxp3 [8]. In 2006, CD127 (α chain of IL-7 receptor) was described and identified as a suitable and more convenient surface marker to substitute intracellular foxp3 for Tregs isolation [9]. To our knowledge, the expression of peripheral $CD4^+CD25^+CD127^-$ regulatory T cells of URSA patients who received paternal lymphocyte active immunization remains scarcely reported. We consider the potential for its use in screening women at high risk of miscarriage and for prophylactic therapeutic measures for prevention of adverse outcomes. The present study was performed to investigate whether PLII of women with URSA could up-regulate the proportion of $CD4^+CD25^+CD127^-$ regulatory T cells in peripheral blood, and provide insights into the correlation between the efficacy of the treatment and different pregnant results.

Materials and methods

Subjects

The retrospective study initially enrolled 202 subjects who were outpatients from Jan 2011

to Oct 2013 of Obstetrics & Gynecology Hospital affiliated to Fudan University, Shanghai, China. All had suffered consecutive miscarriages of unexplained etiology at least twice during first trimester. There was no successful pregnancy record with the same partner before treatment. Any possible known causes or risk factors account for RSA were verified and excluded by inquiring history and screening a series of routine laboratory and gynecological examinations, which included: ① ultrasound scans, hysterosalpingogram or hysteroscope for anatomic abnormalities of the uterus; ② parental karyotypes determined from peripheral leukocytes to exclude fetal chromosomal aberrations possibility; ③ blood group and antibodies for maternal-fetal ABO incompatibility; ④ antiphospholipid antibodies and D-dimer (DD) were analyzed for autoantibodies and hypercoagulability; ⑤ analysis for sperm quality of husbands; ⑥ microbiological investigations for intercurrent infection if highly suspected; ⑦ hormonal profile for endocrinological abnormalities if clinically needed. After screening, altogether 168 cases with complete follow-up data were eligible for further study.

All 168 patients underwent PLII in order to prevent future abortions. The protocol followed was approved by the institutes and ethical committee of the hospital. Before treatment, husbands were required to scan for HIV and hepatitis markers to promise the security of cell transfusion. All of the patients enrolled were explained the purpose, commitment, course of treatment and signed a written informed consent upon the point of the first treatment. Detection of BA by MLC-BE and BE-Ab2 as well as expression of peripheral $CD4^+CD25^+CD127^-$ regulatory T cells were done both before and after lymphocyte immunization four times.

One way mixed lymphocyte culture (MLC)

The efficiency of blocking antibody was evaluated by one-way MLC. Blood was collected under sterile condition; mononuclear leucocytes were isolated from heparinized whole blood, prepared by density gradient centrifugation on Ficoll-hypaque gradient. Mitomycin (Kyowa Hakko Kirin Co Ltd., Tokyo, Japan) treated paternal stimulate cells were divided into three groups for one way mixed lymphocyte culture: (A) cultured with maternal serum for one hour, after washing, add 25% fetal bovine serum; (B) as the control group, add 25% fetal bovine serum; (C) incubated in the 25% maternal serum. All the above cell groups were cultured in round bottomed 96 well plates with the maternal responder cells at a concentration of 2×10^6 cells/ml respectively. Plates were kept at 37°C in a 5% CO_2 atmosphere. Proliferation was measured at day 5 with 0.6 μCi/well of ^3H thymidine (China Isotope Corporation, Beijing, China) incorporation in the last 18 hrs before harvesting. The radioactivity was measured by liquid scintillation counting (MicroBeta Trilux). The percentage (%) of inhibition was calculated by the following formula (cpm = count per minute): MLC-BE %Inhibition = (1 - cpm in A group/cpm in B group) ×100%; BE-Ab2 %Inhibition = (cpm in C group - cpm in A group)/cpm in B group ×100%; %Inhibition ≥5% was defined MLC-BE or BE-Ab2 positive; <5% was defined negative.

FCM for detection of $CD4^+CD25^+$ $CD127^-$ regulatory T cells (Tregs)

Evaluation peripheral expression of $CD4^+CD25^+$ $CD127^-$ regulatory T lymphocyte cells was performed using a standard technique of immunofluorescent marking of heparinized full blood. ① Staining: test tube was added with 50 μl heparin anticoagulated venous complete blood, then 10 μl 1:2 pre-diluted PerCP-Cy5.5-conjugated CD3, FITC-conjugated anti-CD4, PE-conjugated anti-CD25 and 10 μl APC-conjugated anti-CD127 monoclonal antibodies (all from BD Pharmingen, San Diego, CA) were added respectively and mixed. ② Hemolysis: After 30 min incubation at room temperature in the dark, erythrocytes were eliminated by incubation with 5 ml ACK (Red blood cell lysis buffer) for additional 15 min and washing. The samples were then centrifuged at 2000 rpm for 5 min at room temperature, and the supernatants were aspirated and deserted. ③ Ablution: The samples were washed with 0.9% saline, the cell pellets were re-suspended in 0.5 ml 0.9% saline. ④ Detection: For the evaluations, the treated cells were analyzed by BD FACSCalibur™ Flow Cytometry System. 10000 cells were counted for each sample. For the analysis, a real-time gate was set around the viable lymphocytes based on their forward and side-scatter profile. The ratio of $CD3^+CD4^+$ $CD25^+CD127^-$ regulatory T cells in lymphocytes was calculated using BD CellQuest Pro software for general data acquisition and analysis.

Table 1. Characteristics of succeeded and failed groups

	Outcomes of pregnancy after immunotherapy				
	Succeed (n=86)		failed (n=31)		
	Range	Mean ± SD	Mean ± SD	t	P
Age of patients	25~39	30.33±3.37	31.16±3.29	-1.191	0.236
Times of abortion	2~8	2.85±0.99	3.13±1.28	-1.246	0.215
Times of treatments*	3~6	3.93±1.38	3.87±1.34	0.207	0.836

Note: Data are expressed as the mean ± SD. The succeeded group versus failed pregnant group: $P>0.05$. *Before pregnancy.

Table 2. Levels of blocking antibody (MLC-BE) before and after treatment of women with different pregnant outcome

Group	Before therapy MLC-BE (%)	After therapy MLC-BE (%)
Succeed (n=86)	-14.16±17.41	12.15±12.09[a]
Failure (n=31)	-12.96±18.83	-6.82±15.70[b]

Note: Data are expressed as the mean ± SD. [a]$P<0.01$, [b]$P=0.05$ compared to before therapy control.

Table 3. Levels of anti-idio blocking antibody (BE-Ab2) before and after treatment of women with different pregnant outcome

Group	Before therapy BE-Ab2 (%)	After therapy BE-Ab2 (%)
Succeed (n=86)	-10.66±12.53	5.75±10.16[a]
Failure (n=31)	-8.70±10.77	0.31±13.42[a]

Note: Data are expressed as the mean ± SD. [a]$P<0.01$ compared to before therapy control.

Isolation of lymphocytes

Alloimmunization was performed according to the principles described earlier [10]. 50 ml heparinized venous peripheral blood was obtained from the husband of an URSA couple in sterile conditions. Lymphocytes were isolated by lymphocyte separation medium (Ficoll-hypaque) (Hua Jing Biological High-Tec Cop. Ltd., Shanghai, China) for centrifugation, and then the cells (LYM) were collected and washed three times with sterile saline.

Preparation of induced fluid

Per 100 ml induced fluid was composed proportionally of 5 ml recombinant human interferon gamma (IFN-γ, 5%), 2 ml phytohemagglutinin (PHA-M) (2%), 20 ml calf serum (20%) and 73 ml RPMI 1640 medium.

Paternal lymphocytes induced immune (PLII)

The isolated LYM were re-suspended and cultured with the medium that was composed of 10 ml RPMI 1640 liquid (Gibco, USA) and 10 ml induced fluid. After cultivation for 72 hours at 37°C in 5% CO_2 atmosphere in Fisher Scientific CO_2 incubator (CA-1390-1), the lymphocytes were washed three times with sterile saline. Any residual amount of erythrocytes was eliminated with sterile distilled water for injection. Then the obtained lymphocytes were re-suspended in 2 ml 0.9% saline at a concentration of 2×10^7/mL. The cell solution was given intracutaneously at the inside of both forearms at 6 spots. An immunotherapy course was comprised of 3 times of immunization at an interval of 3-4 weeks. Usually one to two courses were needed prior to conception and then continue until over 16th gestational weeks. Levels of MLC-BE, BE-Ab2 and peripheral $CD4^+CD25^+CD127^-$ were again analyzed after four times of immunization.

Patient follow-up

Follow-up revealed that of the enrollment 138 couples were conceived again, of which 86 were successfully pregnant to over 20 weeks of gestation or gave birth to live newborns, 31 cases again with fetus loss despite the treatments, 21 cases conceived babies less than 20 weeks were under follow-up; another 30 cases were still in the state of unpregnancy.

Statistical analysis

All statistical analyses were processed by SPSS 17.0 statistical software, conducted by two independent samples *t*-test, and paired samples *t*-test respectively. Data were presented as mean ± SD (standard deviation). Comparison of MLC-BE and BE-Ab2 positive rates between the two groups was processed by using paired X^2 test. Kappa coefficient was valued in consis-

Table 4. Pregnant outcome of MLC-BE positive and negative group after paternal lymphocyte immunization in URSA patients

		Outcome of pregnancy			
	group	Succeed	Failure	total	Successful rate
MLC-BE after treatment	positive	57	11	68	83.8%
	negative	29	20	49	59.2%*
	total	86	31	117	73.5%
MLC-BE Positive rate		66.3%	35.5%	58.1%	

MLC-BE positive group vs. negative group after immunization (*P<0.05).

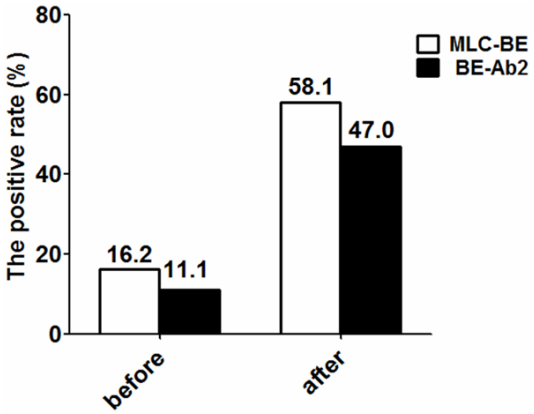

Figure 1. The positive rate of MLC-BE and BE-Ab2 before and after PLII.

tency check for two diagnostic tests. P value of <0.05 was adopted as statistically significant.

Results

PLII significantly elevated the level of MLC-BE and BE-Ab2 of URSA patients

After nearly three years of follow-up, 138 of all 168 studying subjects were conceived again after PLII treatments, of which 117 had already known the final outcome of pregnancy, 21 cases already conceived were under continuing follow-up, another 30 had not attained conception. For further study, 86 resulted in live birth or ongoing gestation of at least 20 weeks were defined to succeeded group, 31 women again lose their fetuses in the first trimester were defined to failed group. Therefore, the total effective rate of PLII was 73.5% (**Table 4**). For detailed further analysis, the 117 women who had already got pregnant with exact outcome were classified into two groups, the succeeded and failed. Age, times of abortion as well as times of treatments of both groups were insignificant (P>0.05), so data were comparable between the two groups (**Table 1**).

Both the level of MLC-BE and BE-Ab2 were significantly elevated in succeeded group after PLII which changed from -14.16±17.41 to 12.15±12.09, and -10.66±12.53 to 5.75±10.16, respectively (P<0.01) (**Tables 2** and **3**). However, a significant elevation of BE-Ab2 was also observed in the failed group, which changed from -8.70±10.77 to 0.31±13.42 (P<0.01) (**Table 3**). The data showed that the increase of BE-Ab2 level in succeeded group was significantly higher than that in failed group. Totally, as shown in **Figure 1**, the positive rate of MLC-BE was raised from 16.2% (19/117) to 58.1% (68/117), and BE-Ab2 from 11.1% (13/117) to 47.0% (55/117), indicating that PLII was actually an effective method to enhance BA level of URSA patients.

Sensitivity and specificity of MLC-BE and BE-Ab2 for evaluating the PLII efficacy in URSA

MLC-BE positive conversion rate after PLII of succeeded and failed groups was 66.3% (57/86) and 35.5% (11/31), respectively. Turn into another perspective angle, accordingly the successful pregnant rate of MLC-BE positive and negative groups in URSA patients was 83.8% (57/68) and 59.2% (29/49), respectively, and showed a statistically significance (P<0.05) (**Table 4**). BE-Ab2 positive conversion rate after PLII of succeeded and failed groups was 50.0% (43/86) and 38.7% (12/31), respectively (**Table 5**). However, the successful pregnant rate of BE-Ab2 positive and negative groups in URSA patients was 78.2% (43/55) and 69.4% (43/62) and also showed no statistically significance (P>0.05) (**Table 5**).

Evaluation the value of MLC-BE and BE-Ab2 to predict the pregnant outcome after lymphocyte therapy for URSA women was revealed in **Table 6**. The sensitivity and specificity of MLC-BE were 66.3% and 64.5% (**Table 6**); as for BE-Ab2, were 50.0% and 61.3% (**Table 6**); as for combi-

Table 5. Pregnant outcome of BE-Ab2 positive and negative group after paternal lymphocyte immunization in URSA patients

		Outcome of pregnancy			
	Group	Succeed	Failure	Total	Successful rate
BE-Ab2 after treatment	Positive	43	12	55	78.2%^{NS}
	Negative	43	19	62	69.4%
	Total	86	31	117	73.5%
BE-Ab2 Positive rate					

BE-Ab2 positive group vs. negative group after immunization ($^{NS}P>0.05$).

Table 6. Evaluation of blocking antibody (MLC-BE) and anti-idio blocking antibody (BE-Ab2) to predict the pregnant outcome after lymphocyte therapy for URSA women

Parameter	sensitivity (%)	specificity (%)	mistake diagnostic rate (%)	omission diagnostic rate (%)	total consistent rate (%)
MLC-BE	66.3	64.5	35.5	33.7	65.8
BE-Ab2	50.0	61.3	38.7	50.0	52.9
MLC-BE+ BE-Ab2	68.3	70.0	30.0	36.6	68.9

Table 7. Consistency check of the two diagnostic tests of blocking antibody (MLC-BE) and anti-idio blocking antibody (BE-Ab2), Kappa =0.251 ($P<0.01$)

		BE-Ab2		
		Positive	Negative	Total
MLC-BE	Positive	38	49	87
	Negative	29	118	147
	Total	67	167	234

Table 8. Proportions of $CD4^+$/LYM in URSA patients of different pregnancy outcome groups before and after immunotherapy

Group	Before therapy CD4+/LYM (%)	After therapy CD4+/LYM (%)
Succeed (n=86)	31.17±6.80	31.23±7.10^{NS}
Failure (n=31)	28.33±6.10	28.27±5.36^{NS}

Note: Values are mean ± SD. $^{NS}P>0.05$.

Table 9. Proportions of $CD4^+CD25^+$/LYM in URSA patients of different pregnancy outcome groups before and after immunotherapy

Group	Before therapy CD4+CD25+/LYM (%)	After therapy CD4+ CD25+/LYM (%)
Succeed (n=86)	10.55±5.78	15.45±6.88^a
Failure (n=31)	13.55±4.08	15.15±5.15

Note: Values are mean ± SD. $^aP<0.01$.

nation analysis of MLC-BE with BE-Ab2, were 68.3% and 70.0% (**Table 6**), respectively. Regard the successful pregnant rate as the "gold standard", the total consistent rate of MLC-BE and BE-Ab2 were 65.8% and 52.9%. In many cases, using the above indicators to predict treatment outcomes would lead to mistake or omission diagnostic which reached up to about thirty to fifty percent as shown in **Table 6**. Consistency check of the two diagnostic tests of MLC-BE and BE-Ab2 clearly showed that there existed a significant correlation between the two parameters ($P<0.01$). However, the Kappa coefficient was only 0.251, which indicated only weak or moderate consistency (**Table 7**). These results suggested that the sensitivity and specificity of MLC-BE and BE-Ab2 for evaluating the PLII efficacy in URSA have certain limitations.

Proportion of peripheral $CD4^+CD25^+CD127^-$ Treg in succeed group was elevated

Then, FCM was performed to analyze the percentage of $CD4^+$ T cell/total peripheral blood mononuclear cell (PBMC), $CD4^+CD25^+$ T cell/PBMC and $CD4^+CD25^+CD127^-$ Treg cell/ $CD4^+$ T cell in peripheral blood of URSA patients with different pregnancy outcome groups before and after alloimmunization. Data were presented in **Figure 2** and **Tables 8-10**, the frequencies of $CD4^+$/PBMC kept the same level at the stage of pre-treatment and post-treatment in both two groups ($P>0.05$) (**Table 8**). After one to two courses of treatments, the percentage of $CD4^+CD25^+$ T cell/PBMC and $CD4^+CD25^+CD127^-$ Treg/$CD4^+$ T cell in succeeded group were significantly elevated from

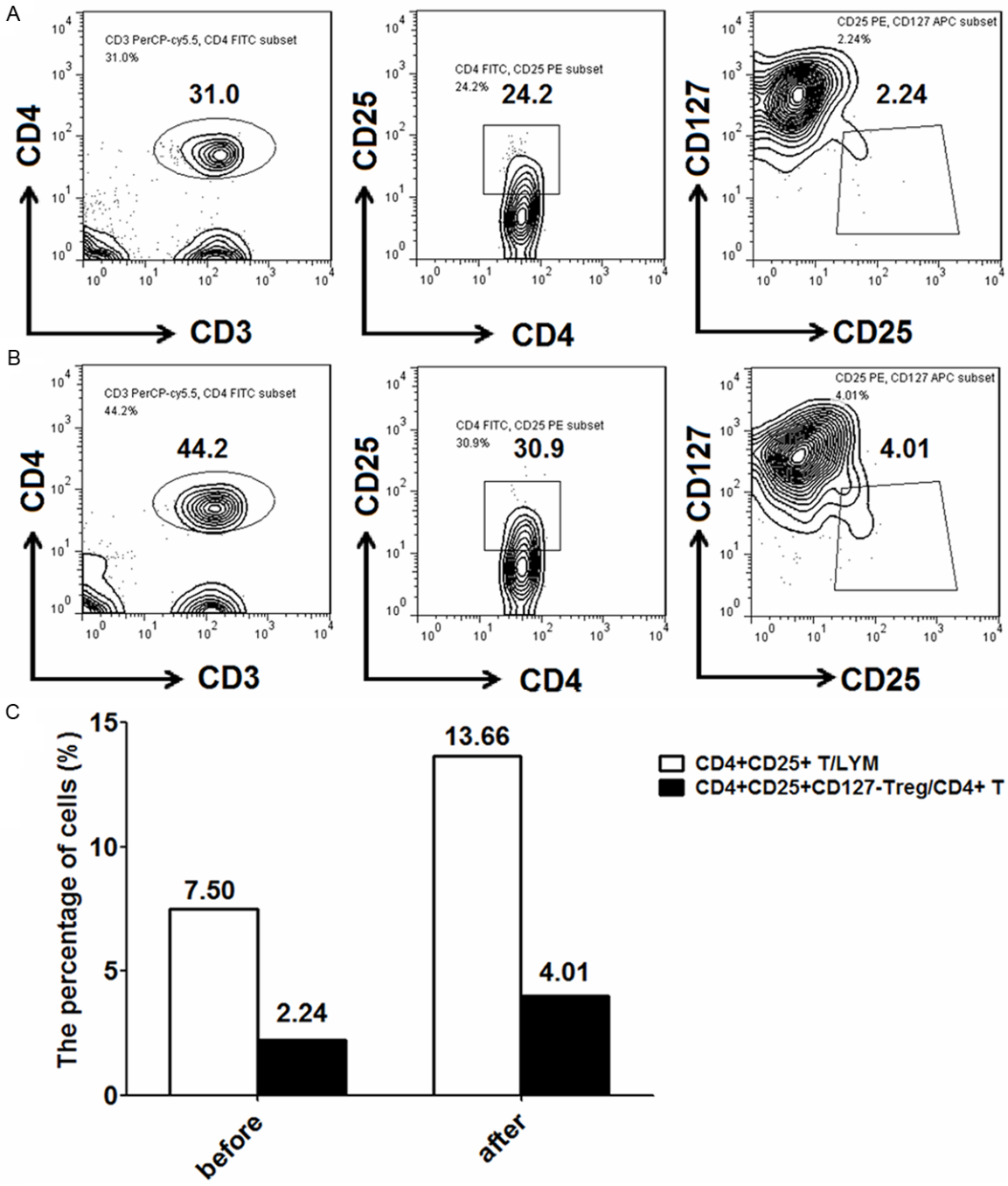

Figure 2. The peripheral ratio of Tregs in URSA women before and after PLII. There were original data of a succeeded case (aged 34, suffered 3 abortions before) both before and after PLII. Peripheral $CD4^+$/LYM (Lymphocytes) kept the same level between pre-treatment (A) and post-treatment (B), while $CD4^+CD25^+$ T/LYM and $CD4^+CD25^+CD127^-$ Treg/$CD4^+$ T cell were obviously elevated from 7.50 to 13.66 and 2.24 to 4.01, respectively (C).

10.55±5.78 to 15.45±6.88 (P<0.01) (**Table 9**) and 1.69±0.90 to 3.48±1.19 (P<0.01) (**Table 10**), respectively. However, in the failed group, we did not observe the significant change of the proportion of $CD4^+CD25^+$ T cell/PBMC and $CD4^+CD25^+CD127^-/CD4^+$ T cell. Moreover, the level of Tregs still maintained a low level, ranged from 13.55±4.08 to 15.15±5.15 (P>0.05) (**Table 9**) and 1.81±0.41 to 2.04±0.68 (P >0.05) (**Table 10**), respectively.

Table 10. Proportions of $CD4^+CD25^+CD127^-/CD4^+$ in URSA patients of different pregnancy outcome groups before and after immunotherapy

Group	Before therapy $CD4^+CD25^+CD127^-/CD4^+$ (%)	After therapy $CD4^+CD25^+CD127^-/CD4^+$ (%)
Succeed (n=86)	1.69±0.90	3.48±1.19[a]
Failure (n=31)	1.81±0.41	2.04±0.68

Note: Values are mean ± SD. [a]$P<0.01$.

Table 11. Bivariate Pearson Correlation analysis of blocking antibody (MLC-BE), anti-idio blocking antibody (BE-Ab2), peripheral $CD4^+CD25^+$,and $CD4^+CD25^+CD127^-$ Tregs (n=234)

	MLC-BE	BE-Ab2	$CD4^+CD25^+$/ PBMC	$CD4^+CD25^+$ $CD127^-/CD4$
MLC-BE	1	0.410*	0.175	0.373*
BE-Ab2	0.410*	1	0.193	0.315*
$CD4^+CD25^+$/LYM	0.175	0.193	1	0.323*
$CD4^+CD25^+CD127^-/CD4$	0.373*	0.315*	0.323*	1

Note: *$P<0.01$, the correlation is significant at the 0.01 level (2-tailed).

Combination analysis of $CD4^+CD25^+CD127^-$ Treg cells with MLC-BE and BE-Ab2 is an efficient evaluation for PLII in URSA

A bivariate Pearson correlation analysis of MLC-BE, BE-Ab2, the percentage of peripheral $CD4^+CD25^+$ T cell/LPBMC and $CD4^+CD25^+$ $CD127^-$ Treg/$CD4^+$ T cell was presented in **Table 11**. Among these, we observed that peripheral $CD4^+CD25^+CD127^-$ Treg/$CD4^+$ T cell was significantly positive related with MLC-BE (r=0.373) ($P<0.01$) and BE-Ab2 (r=0.315) ($P<0.01$).

In order to further evaluate the value of combination analysis of $CD4^+CD25^+CD127^-$ Treg cells with MLC-BE and BE-Ab2 (Treg+MLC-BE+BE-Ab2) in predicting the pregnant outcome, we counted the sensitivity and specificity of Treg (1.5) (The ratio of peripheral $CD4^+CD25^+CD127^-$ Treg/$CD4^+$ T cell before PLII/after PLII ≥1.5), Treg (2.0) (The ratio of peripheral $CD4^+$ $CD25^+CD127^-$ Treg/$CD4^+$ T cell before PLII/after PLII ≥2.0), Treg (1.5)+MLC-BE+BE-Ab2, and Treg (2.0)+MLC-BE+BE-Ab2, and found that combination analysis of Treg (1.5) has the highest sensitivity (81.8%) and specificity (81.3%) for evaluating the efficiency of PLII in URSA (**Table 12**). These results suggest that the combination analysis of $CD4^+CD25^+CD127^-$ Tregs with MLC-BE and BE-Ab2 may be an efficient evaluation of the therapy of paternal lymphocyte induced immunization in URSA patients.

Discussion

RSA with unexpected cause is a heterogeneous condition, for which immunological disorder accounts for nearly half of the etiology. Considerable knowledge on the mechanisms leading to feto-maternal interface tolerance had been published in recent decades. Blocking antibody (BA), also defined as anti-paternal lymphocyte antibody (APLA), is IgG3 in nature produced by maternal immune system stimulated by HLA, trophoblast and lymphocyte cross-reactive antigen. Lack of BA proved to be the main cause of miscarriage with unexpected reasons. The inhibition mechanism of BA includes: neutralizing paternal alloantigen to block antigen recognition which consequently protects the fetus from rejection; decreases of the percentage of natural killer cells in peripheral blood in women with RSA and motivation of a shift of the immune balance from Th1 type toward Th2 type which contribute to the maintenance of pregnancy. Consensus have been reached that the increment of compatibility between HLA loci of the couple leads to inability of proper recognition of fetal alloantigens by the maternal immune system ,and this situation is subsequently associated with recurrent pregnancy loss. Behar *et al.* assessed peripheral blood lymphocytes from habitually aborting women before and after immunization and found that PLII may induce specific and non-specific T-cell suppression which may, in turn, induce the immune tolerance of conceptus antigens which is necessary to maintain pregnancy [10]. The rationale for this treatment was to create anti-paternal cytotoxic or blocking antibodies which were convinced to prevent miscarriage. Over past decade in China, lymphocyte active immunization has become one of the most widely performed scheme for alloimmune-mediated miscarriages, though the protocols of different medical organizations are not quite unified yet.

However, although there is a general trend favoring PLII, the therapy still remains contro-

Table 12. Evaluation of Treg ratio, blocking antibody (MLC-BE) and anti-idio blocking antibody (BE-Ab2) to predict the pregnant outcome after lymphocyte therapy for URSA women

Parameter	Sensitivity (%)	Specificity (%)	Mistake diagnostic rate (%)	Omission diagnostic rate (%)	Total consistent rate (%)
MLC-BE+ BE-Ab2	68.3	70.0	30.0	36.6	68.9
Treg (1.5)	69.8	80.6	19.4	30.2	72.6
Treg (2)	51.1	96.8	3.2	48.8	63.2
Treg (1.5) + MLC-BE+ BE-Ab2	81.8	81.3	18.8	18.2	81.6
Treg (2) + MLC-BE+ BE-Ab2	72.2	92.8	7.1	27.8	81.3

Note: Treg (1.5): The ratio of peripheral $CD4^+CD25^+CD127^-$ Treg/$CD4^+$ T cell before PLII/after PLII≥1.5; Treg (2): The ratio of peripheral $CD4^+CD25^+CD127^-$ Treg/$CD4^+$ T cell before PLII/after PLII≥2.

versial in terms of its effectiveness. A few reports argued that lymphocyte therapy did not lower the risk of future miscarriage [11]. Sargent *et al.* discovered no association between the level of one-way MLC and pregnancy outcome in normal and RSA women. Pena *et al.* reported that the alloimmunization induced MLC-BE in women with RSA was not associated with successful outcome of pregnancy [12]. Correspondingly, a great deal of uncertainty and confusion exists regarding the choice of a marker for evaluating efficacy of immunotherapy for treatment of RSA. Some studies have found that so-called blocking antibodies have no predictive value with respect to pregnancy outcome [13]. Dispute with the above conclusions, many researches here in China support the view that lymphocytes immunization exerts a pivotal role in maintaining successful pregnant outcome by improving the level of BA. It is noteworthy that most of these publications reported the results of non-induced paternal lymphocyte immunization [14].

Our laboratory has been using *in vitro* induced lymphocyte immunotherapy for URSA women for more over 15 years [15]. Consistent with other reports, the present investigation also revealed satisfied clinical efficacy of allo-immunization which totally was 73.5%. Detailed analysis show 57 of 68 patients (83.8%) who were seroconverted had gestational success, while 29 of 49 (59.2%) of those who were BA negative had also shown successful pregnant results. Anyway, the successful rate of the two groups show significantly difference, indicating there existed a positive correlation relationship between MLC-BE level and successful pregnancy results to some extent. But apparently, regardless of the significance described above, MLC-BE was neither sensitive nor specific (**Table 6**), as even in the BA negative group after immunization, the successful rate was as high as nearly 60%; vise versa, in the still failed pregnant group , 35.5% (11/31) patients were MLC-BE positive as presented in **Table 5**. It can be concluded that the successful pregnant rate of BE-Ab2 positive and negative groups after lymphocyte immunization in URSA patients were 78.2% (43/55) and 69.4% (43/62), respectively, and showed no significance (**Table 5**). Overall, we found that PLII indeed elevated the level of BA and anti-idiotypic antibody significantly, neither MLC-BE nor BE-Ab2 was a very ideal marker for predict the treatment effect. Therefore, search for new biomarkers to help better predict efficacy of treatment is of great importance.

Originally discovered by Gershon in 1970, Treg is a rare cell population representing approximately 5-10% of all $CD4^+$ cells [16]. These immuno-regulatory cells are thought to play a critical role in the control of T-cell mediated autoimmunity by suppressing the proliferation and cytokine production of other T cells. A considerable number of studies in the past few decades had been dedicated toward Tregs, which is highlighted by their significantly protective effect for induction and maintenance of fetal-maternal immunologic tolerance. It has been proven that Tregs expand during pregnancy and are crucial for the maintenance of a physiological pregnancy [17]. It is well recognized from previous research that decreased frequency and deficient function of Tregs result in pathological conditions such as URSA. Previous studies had demonstrated that Tregs can be activated and expanded *in vitro*, and activated Tregs usually secrete high levels of inhibitory cytokines, such as IL-10 and TGF-β1.

Yin *et al*. reported that adoptive transfer with *in vitro* expanded Tregs significantly reduced the fetal resorption rates of a mice model of spontaneous abortion at early stage of pregnancy. Furthermore, there existed the increased levels of serum IL-10, TGF-β1, and the increased ratios of IL-10 to IFN-γ [18]. The discovery of Foxp3, a transcription factor, as a specific marker of Tregs expanded research in biological properties of these cells, and is considered to be among the currently most accepted definitive marker associated with Tregs [19]. However, Foxp3 is an intracellular expressed molecule, so its detection process requires punching and permeabilizing the cell membrane, the relatively sophisticate and cost procedure prevent the use of these cells in downstream applications as well as in the field of biology and clinical research. CD127 expressed highly and inversely correlates with Foxp3 and suppressive function of human $CD4^+$ Treg cells [20]. Therefore, the detection of $CD4^+CD25^+$ $CD127^-$ cells level has been widely recommended to allow consistent identification and sorting for live Tregs more accurately and conveniently.

To our knowledge, there is scarce data available on the reports refer to level of $CD4^+CD25^+$ $CD127^-$ Tregs in peripheral blood in women with URSA before and after immunotherapy. In the present study, a significant expansion of Tregs indicating alteration of peripheral tolerance was seen after lymphocyte immunotherapy. We observed significant elevated proportional Tregs level in those who successfully maintained pregnancy over 20 gastational weeks after alloimmunization (from 1.69±0.90 to 3.48±1.19). In contrast, in those who failed again, the expression still kept relatively low level despite existence of slightly change from 1.81±0.41 to 2.04±0.68, $P>0.05$) (**Table 10**). The present data suggest that immunization induced up-regulation of $CD4^+CD25^+$ $CD127^-$ Tregs in peripheral blood play a vital role in maintaining maternal tolerance of the allogeneic fetus, contributing to create better prognosis for the outcome of the pregnancy for women with RSA of unknown etiology. A Bivariate Pearson correlation analysis indicated that peripheral $CD4^+CD25^+CD127^-$ Tregs correlated well with MLC-BE and BE-Ab2. Further investigation for the mechanism of correlation and possible interplay between BA and peripheral percentage of $CD4^+CD25^+CD127^-$ Tregs were to be studied.

In summarize, we speculate the change of peripheral $CD4^+CD25^+$ $CD127^-$ Tregs provides a novel convenient and feasible screening method for determining women at high risk of abortion, and may help design effective treatment strategies for this intractable disease. Our recommendation is that at the present stage, combined use of peripheral $CD4^+CD25^+CD127^-$ Tregs with MLC-BE and BE-Ab2 should be more persuasive and accurate for diagnosis and evaluation of the immunotherapy than only dependent on the conventional commonly used BA parameter alone.

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Disclosure of conflict of interest

None.

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